

STUDIES ON THE SYNTHESIS AND FUNCTIONS
OF IMMUNOGLOBULINS IN THE FOWL

by

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SUMMARY

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The immunoglobulins of the chicken have been studied with particular reference to their synthesis and function.

Since information on the physico-chemical properties of normal immunoglobulins in the fowl was limited, the initial part of the work was concerned with the production of specific anti IgG and anti IgM antisera and further characterisation studies of these proteins including gel filtration and DEAE cellulose chromatography. Attempts were made to isolate other immunoglobulin classes and a third component - referred to as 7S γ 1 - was studied and some of its physico-chemical properties noted. It was not possible either by radioimmuno-electrophoresis or antiglobulin studies to demonstrate antibody activity in this serum component. Later, analyses of sera from bursectomised chickens where normal amounts of 7S γ 1 were present also indicated that this was not an immunoglobulin.

The experimental procedure of bursectomy was used to study immunoglobulin synthesis and function. A method for the surgical removal of the bursa of Fabricius in ovo was developed and is described in detail.

Serum IgG and IgM levels in normal birds and birds bursectomised as 17, 18 or 19 day old embryos were measured from hatching to adulthood and three distinct patterns of development were noted in the bursectomised birds - they were either deficient in both immunoglobulins, deficient in IgG with higher than normal levels of IgM or produced substantial amounts of both Ig types. IgG and IgM levels

at 3 months of age were compared for birds bursectomised at 17, 18 or 19 days and a significant difference was noted in the numbers of birds deficient in IgM in the 17 day group as compared with the others. All groups showed marked inhibition in the development of IgG synthesis. These results are discussed in the light of current theories of the mechanism of development of immunocompetence.

A long-term study of serum immunoglobulins in bursectomised birds showed an eventual decline to agammaglobulinaemia even in those birds with normal initial immunoglobulin levels.

The survival rates of bursectomised birds are compared with controls and studied in relation to immunoglobulin status. Possible reasons for differences noted in survival are suggested.

Specific antibody responses of normal and bursectomised birds to bovine serum albumin, sheep erythrocytes and live Salmonella gallinarum were measured and related to immunoglobulin synthesis.

The receptor function of immunoglobulin molecules on the surfaces of lymphocytes has been investigated. Immunoglobulin determinants have been demonstrated on the surface of bursa dependent lymphocytes but not thymus dependent lymphocytes by the mixed antiglobulin method. These results indicate that the surface receptors of "T" lymphocytes may not be immunoglobulin in nature, and possible methods for the detection and characterisation of these

receptors are suggested, and initial attempts to do so described.

The literature on avian immune responses is reviewed and the most important findings in the present work discussed. Suggestions are made for further research in various areas of the work.

SECTION I

GENERAL INTRODUCTION

STUDIES ON CHICKEN ANTIBODIES

The immune response in chickens has been studied in detail only during the last few decades. The earliest report on precipitin production by chickens appeared in 1903 by Ewing and Strauss whose interest was in the medico-legal use of fowl antisera. Other workers made similar use of chicken sera and in 1913 Sutherland and Mitra published an account of some misleading precipitation reactions which could occur. They noted that antisera could be made more specific for human blood by diluting out activity against other mammalian species and that this was more effective if the dilutions were made with greater than physiological concentrations of salt. Similar findings were reported by Hektoen, (1918) who also included details of immunisation schedules and precipitin titres.

20 years later Wolfe and his colleagues embarked on a detailed study of precipitin production in the chicken and the effects of physical factors on the precipitation reaction (Wolfe, 1942; Goodman et al., 1951; Gengozian and Wolfe, 1957; Banovitz and Wolfe, 1959). They found, as the earlier workers had, that chickens were very efficient producers of precipitating antibodies but that these antibodies differed from mammalian ones in several respects.

The most striking was the effect of varying concentrations of NaCl on precipitate formation

(Goodman et al., 1951). Precipitation of antigen by chicken antibody increased as the salt concentration was increased above the normal physiological level of 0.15 M to reach a maximum at 1.5 M NaCl. Thus determinations of maximum precipitin content of chicken sera must be carried out at a salt concentration of at least 8%.

Ageing of the antiserum also affected its precipitation properties (Wolfe, 1942; Makinodan et al., 1960), causing a decrease in the amount of precipitate formed with antigen in 1.5 M NaCl, but an increase in precipitation in 0.15 M NaCl.

Makinodan et al., (1960) showed that a normal serum macroglobulin protein coprecipitated with the antigen-antibody complex in 1.5 M NaCl regardless of the age of the antiserum, but that coprecipitation in 0.15 M NaCl occurred only with aged and not with fresh antiserum. Reports by Deutsch et al., (1949) of coprecipitation of α_1 -globulins with antigen-antibody complexes have not been confirmed by others (Goodman and Ramsey, 1957; Banovitz et al., 1959) but Van Orden and Treffers (1968) found coprecipitation of β -lipoproteins with specific precipitates of chicken antibodies and human serum albumin in 8% NaCl.

More recent workers have been concerned with the isolation of specific antibodies and the study of their physico-chemical properties. Orlans et al., (1961) reported that the antibodies produced in response to

several different antigens were of two distinct types, both of γ electrophoretic mobility but differing from each other antigenically and in molecular weight. One type had a molecular weight of 180,000, the other 600,000. Both types precipitated in both 0.15 M and 1.5 M NaCl but the precipitate formed at the high salt concentration contained more of the lower molecular weight antibody.

Benedict et al., (1963), characterising chicken anti-bovine serum albumin (BSA) antibodies by chromatography and ultracentrifugation, showed the presence of both macroglobulin type and low molecular weight (7S) antibodies. Riha, (1965) also found that macroglobulin and 7S antibodies were produced against the same antigenic determinants, but reported that the specificity and firmness of binding of macroglobulin antibody was lower than that of the 7S type.

Gallagher and Voss, (1969) found the molecular weight of a purified chicken anti-dinitrophenyl (DNP) antibody to be 178,000 - 179,000, a value which is in agreement with the findings of the previous mentioned authors. On the other hand, Tenenhouse and Deutsch (1966) found chicken 7S γ -globulins to have a molecular weight of around 206,000. However, these were normal serum immunoglobulins as opposed to the purified specific antibodies studied by others and may have been a heterogeneous population.

Other properties of chicken antibodies have been investigated to some extent. These include the following:

1. electrophoretic mobility, which is higher than that of mammalian immunoglobulins in barbital buffer pH 8.6 (Nichol and Deutsch, 1948; Tenenhouse and Deutsch, 1966);
2. digestion by papain and pepsin, (Tenenhouse and Deutsch, 1966; Dreesman and Benedict, 1965a; Kubo and Benedict, 1969);
3. mercaptoethanol (ME) sensitivity. There is some controversy about this property, Szenberg et al., (1965) reporting that the titre of 7S antibodies as well as the macroglobulin type is considerably reduced by ME treatment, whilst Benedict et al., (1963) and Rosenquist and Campbell, (1966) found that 7S antibody retained its activity after treatment;
4. carbohydrate content, reported variously as being 3.1% (Tenenhouse and Deutsch, 1966) and 2.5% (Benedict, 1967) for 7S antibody;
5. chromatography characteristics (Benedict et al., 1963; Szenberg et al., 1965);
6. amino-acid analyses (Tenenhouse and Deutsch, 1966; Dreesman et al., 1965).

Some of these aspects will be discussed in more detail in a later section.

IMMUNOGLOBULIN CLASSES

In spite of the above mentioned studies, far less is known about the immunochemistry of avian immunoglobulins than about their mammalian counterparts.

At this point it seems relevant to include a short account of the mammalian immunoglobulins as a background against which to discuss chicken immunoglobulin classes.

Mammalian Immunoglobulins: The structure and immunochemistry of mammalian immunoglobulins has been reviewed by several authors, (Cohen and Porter, 1964; Edelman and Marchalonis, 1967). A World Health Organisation Committee in 1964 defined immunoglobulins as "proteins of animal origin endowed with known antibody activity and certain proteins related to them by chemical structure and hence antigenic specificity", and proposed that the standard nomenclature for the main classes ^{in humans} should be IgG, IgM and IgA. IgG is the classical precipitating antibody of molecular weight about 150,000 and with a sedimentation coefficient of 7S. IgM is the macroglobulin (19S) antibody with a molecular weight of about 1,000,000. IgA is a non-precipitating antibody of molecular weight 160,000, and is the antibody of the secretions of the intestine, respiratory tract, saliva, tears and colostrum.

All immunoglobulins are composed of light and heavy polypeptide chains. The light chains are of two types (κ and λ) and are common to all the classes, whilst the heavy chains are antigenically distinct for each class

and are called γ , μ and α respectively. The IgG molecule is composed of two light chains and two heavy γ chains linked together by disulfide and weak hydrogen bonds. When split by papain and reducing agents two Fab (antibody binding) fragments each consisting of one light chain and part of one heavy chain, and one Fc (crystallisable) fragment, consisting of part of each heavy chain, are produced. IgM and IgA molecules are built up from similar basic four chain units, IgM consisting of 5 sub units. IgA molecules in secretions, but not in serum, are dimers and have an extra structure attached to them called a 'secretory piece', the function of which is uncertain. In addition to these three major classes, two more classes have been isolated in human serum, designated IgD and IgE. Sub classes have also been described in several species including human where IgG, IgM and IgA all have sub classes. Bovines, guinea pigs and mice all have various IgG sub classes.

Chicken Immunoglobulins: In spite of several differences between the main precipitating 7S antibody in chicken serum and mammalian IgG, (Tenenhouse and Deutsch, 1966; Leslie and Clem, 1969), notably a higher hexose content, molecular weight, and electrophoretic mobility, most workers now classify chicken immunoglobulins as IgG and IgM. The suggestion by Tenenhouse and Deutsch, (1966) that normal chicken γ globulins are more akin to mammalian IgA than IgG and should be classified as such

does not seem justified (Leslie and Clem, 1969) and has not been generally accepted.

For the purpose of this thesis, the chicken immunoglobulin which resembles mammalian IgG in its immunoelectrophoretic pattern will be called IgG. It gives a single long arc on immunoelectrophoresis which is slightly shorter than mammalian IgG but has the same shape. The $S_{20,w}$ is approximately 7 and the molecular weight about 180,000 (Orlans et al., 1961). Like mammalian IgG it can be split by papain and a reducing agent into Fab and Fc fragments. According to Dreesman and Benedict, (1965b) the data available on the chain structure fits the four chain model described for mammalian IgG.

Chicken IgM also resembles its mammalian counterpart on immunoelectrophoresis, giving a short "gull-shaped" arc extending from the well into the γ_2 area. Ultra-centrifugal analyses give $S_{20,w}$ values of 16.6 and 26-28 (Benedict, 1967) and reduction by 0.1 M mercaptoethanol yields 7S, 5S and 3.6S subunits. The molecular weight is around 890,000.

It seems very probable that as well as these two classes of immunoglobulin several other as yet unrecognised classes exist, in view of the complexity of lines in the γ region on immunoelectrophoresis. There are, in fact, several reports of a third antibody type (Dreesman et al., 1965; Orlans, 1968; Wilkinson and French, 1969; Kono et al., 1969; Ivanyi et al., 1966; Patterson et al., 1965)

although to date no other immunoglobulin class has been isolated and clearly defined.

Certainly many of the variations in the properties of chicken antibodies reported in the literature can be explained by the existence of two distinct immunoglobulin types, both of which appear in the second sephadex G-200 (7S) peak. These discrepancies include:

- (a) the different sedimentation coefficients of 6-9S and 13-16S in 0.15 and 1.5 M NaCl respectively of fowl γ -globulins examined in the ultracentrifuge by Hersh and Benedict, (1966) and Orlans, (1968);
- (b) the unusually high molecular weight of 206,000 recorded by Tenenhouse and Deutsch, (1966);
- (c) the variation in the ability of chicken 7S antibody to cause passive haemagglutination and to precipitate with antigen in 0.15 M NaCl, and
- (d) its variable sensitivity to ME treatment.

These points have been discussed by Orlans, (1968) who also described a 7S anti-DNP antibody which differed from IgG in its extinction coefficient, and by the fact that it precipitated with antigen in 0.9% instead of 8% NaCl, agglutinated antigen-coated tanned red cells, did not fix complement, and possessed skin sensitising properties. This antibody proved difficult to isolate and was neither characterised nor classified.

The other reports of a third avian antibody type, tentatively suggested to be of the IgA class (Kono et al.,

1969; Dreesman et al., 1965; Patterson et al., 1962; Ivanyi et al., 1966; Wilkinson and French, 1969) are based on radioimmuno-electrophoresis studies of serum or egg yolk antibodies, or straightforward immuno-electrophoresis of serum and faecal extracts. In each case the immunoglobulin was not isolated.

CHARACTERISTICS OF IMMUNE RESPONSES IN CHICKENS

Humoral Responses: The characteristics of humoral responses in the chicken, such as time sequences, have been fairly well documented. As already mentioned chickens respond to intravenous injection of protein antigens by producing high levels of precipitating antibodies. Detectable antibody appears as early as 72 hours after a dose of 40 mg soluble BSA (Benedict et al., 1963). A peak titre is reached at between 6 and 10 days (Benedict et al., 1963; Steinberg et al., 1970), after which the antibody level in serum falls rapidly and is at a very low level by day 18.

During a secondary or tertiary response the induction period is shorter but the peak attained is no higher than in a primary response (Benedict et al., 1963).

If the same antigen is given intramuscularly with an adjuvant such as Freund's complete adjuvant, this early antibody curve is not altered. However a second rise in antibody production occurs starting about the 21st day, with a peak between 6 and 7 weeks after antigenic stimulation (French et al., 1970). This second peak only occurs when adjuvant has been used and can reach a level 100 times higher than the first peak. The avidity of

the antibody present at this time is generally much greater than early in the response.

The sequential synthesis of IgM and IgG in chickens during primary and secondary responses to protein antigens appears to follow a pattern similar to that reported for rabbits (Benedict et al., 1963; Dreesman et al., 1965) - that is an early appearance of IgM antibody followed rapidly by a changeover to IgG production. This sequence does not occur, however, during all immune responses, for the type of antibody formed depends to some extent at least, on the nature of the antigen.

Thus Riha found that, after immunisation with p-azobenzoic acid-human serum albumin (p-ABA-HSA), antibodies against the carrier protein appeared early and were of both IgM and IgG types, whereas antibodies to the hapten appeared later and were exclusively macroglobulin (Riha, 1965). Anti-bacteriophage antibodies are also mainly IgM during both primary and secondary responses (Benedict, 1967; Uhr et al., 1962).

Dreesman et al., (1965) detected IgG antibodies and a type tentatively called IgA, but no IgM in all antisera against the hapten azobenzoate, whilst the anti-protein activity was found in the γ G-, γ M- and occasionally γ A- globulins.

Duffus and Allan, (1968) reported an early production of IgM accompanied and followed by increasing amounts of IgG antibodies against the O antigen of S.gallinarum in

chickens immunised with the whole organism.

Cellular Responses: Normal chickens have been shown to be capable of mounting cellular immune reactions identical to those seen in the mammal (Lipton and Steigman, 1961; Warner et al., 1962; Jankovic and Isvaneski, 1963; Jankovic et al., 1963; Warner, 1965; Perey et al., 1970a; Warner et al., 1971). Delayed hypersensitivity reactions to several antigens were shown by Warner et al., (1971) to fulfil all the criteria applied to this type of response in mammals - that is a time-lapse of 24 hours between antigen administration and maximum response, the infiltration into the lesion of large numbers of small lymphocytes along with some granulocytes and macrophages, carrier specificity when responses are induced to hapten-protein conjugates, and in vitro inhibition of the migration of macrophages from sensitised birds by the presence of specific antigen.

Homograft rejection occurs within 8 days in normal chickens (Warner and Szenberg, 1962; Kelly and Abramoff, 1969; Perey et al., 1970a), and cells from blood, spleen and thymus cause graft versus host reactions demonstrable by the production of foci on the chorioallantoic membranes of 12 day old chick embryos (Warner et al., 1962) or splenomegaly in chick embryos which have been inoculated with the cells intravenously (Dent and Good, 1965).

Experimental allergic encephalomyelitis can be induced in birds as in mammals by the injection of nervous

tissue along with adjuvant (Lipton and Steigman, 1961; Jankovic and Isvaneski, 1963). This disease is generally believed to be of the cell mediated delayed hypersensitivity type (Waksman, 1959).

Passive Immunity: It has been known since the beginning of the century that antitoxins are transferred from the sera of vaccinated hens to their egg yolks (Klemperer, 1893; Dziergovski, 1901). Immunity to virus and bacterial infections has also been shown to be transferred passively from the hen to the young chick via the yolk (Brandly et al., 1946; Buxton, 1952; Burmeister, 1955; Malkinson, 1965; Stedman et al., 1969). Little is known, however, of the types of immunoglobulin involved in this transfer. IgM is not implicated since this class of immunoglobulin is not present in egg yolk (Wilkinson and French, 1969) nor in day old chicken serum. Wilkinson and French, (1969) have shown the presence of two antigenically distinct 7S antibodies in yolk, and Buxton, (1952) demonstrated both agglutinating and non-agglutinating antibodies to S.gallinarum in the yolks of eggs from immunised birds. There was often proportionally more of the non-agglutinating type in yolk than in the hens' sera. Orlans, (1967) found that the antibodies against foreign erythrocytes transferred to yolk and young chicks were also mainly non-agglutinating and non-haemolytic.

The time relationship between the appearance of antibody in serum and its transfer to yolk has been studied

by Patterson et al., (1962) using I^{131} labelled BSA as antigen. They found that antibody appeared in the yolk 7-8 days after immunisation of the hen and 4 days after it had first appeared in the serum. When a peak titre was reached in the yolk, the serum antibody level had already started to fall.

THE TISSUES INVOLVED IN IMMUNE RESPONSES

The lymphoid organs in the chicken consist of the spleen, bone marrow, caecal tonsil, thymus, and an organ unique to avian species, the bursa of Fabricius. This is an unpaired sac-like organ situated on the dorsal wall of the cloaca and connected with the cloacal cavity by a small duct. It develops before the 12th day of embryonation as a follicular epithelial organ and becomes lymphoid between the 13th and 21st day, the lymphoid follicles consisting of a cortex and medulla. Like the thymus the bursa starts to involute at about 5 months of age when chickens reach sexual maturity.

Lymph nodes as seen in mammals are absent in the domestic chicken (Gallus domesticus), certain marsh and sea birds (Lamellirostres and Palmipedes) being the only avians to possess these organs and then only as a cervico-thoracic and a lumbar pair of nodes (Jolly, 1909). However, aggregates of lymphoid tissue do occur in the chicken along the course of the lymphatic vessels, either as small infiltrations in the vessel wall, or as

unencapsulated but distinct nodules (Biggs, 1956, 1957). In addition lymphoid tissue can be found dispersed in non-lymphoid organs such as lungs, liver, pancreas, adrenals, ileum and colon, and indeed in any area of connective tissue (Biggs, 1956).

Bang and Bang, (1968) have reported the consistent finding of lymphoid elements in the upper respiratory tract of the domestic chicken and other avian species. In particular the Harderian glands of the nictitating membrane always contained large numbers of plasma cells, whilst the lacrimal gland and duct showed extensive infiltrations of lymphocytes and germinal centres even in chickens raised under germ-free conditions.

Not all the lymphoid tissues have been shown to be involved directly in immune responses. Antibody producing cells occur mainly in the spleen (Makinodan et al., 1954; Jankovic and Mitrovic, 1967; Keily and Abramoff, 1969; Mueller et al., 1971), and following splenectomy (that is complete splenectomy without the spillage of cells into the peritoneal cavity and consequent development of accessory splenules) there is a delay and an overall reduction in antibody responses to the intravenous injection of foreign erythrocytes (Rosenquist and Wolfe, 1962; Graetzer et al., 1963; Keily and Abramoff, 1969).

Antibody is also produced in the bone marrow in normal chickens after intravenous inoculation of erythrocytes and this is suggested by Keily and Abramoff,

to be the major site of antibody production in splenectomised birds.

Other sites where lymphoid tissue is present - lung, liver, caecal tonsil, bursa and thymus - have been examined for antibody producing cells by several workers with mixed results. Keily and Abramoff, (1969) failed to find antibody producing cells in any of these organs after a single intravenous injection of antigen, using Jerne's plaque technique (Jerne and Nordin, 1963). Mueller et al., (1971) using the same technique also failed to show plaque forming cells (PFC) in the caecal tonsil after one intravenous injection of sheep red cells. Jankovic and Mitrovik on the other hand (1967), using the fluorescent antibody technique, demonstrated antibody containing cells in the thymus, caecal tonsil, ileum, colon and caecum, but not in the bursa, following two intravenous injections of BSA. They suggest that the gut-associated lymphoid tissue is an important site of production of antibody in response to the many antigens assailing the body via the digestive tract.

Following the discovery by Bang and Bang, (1968) of lymphoid elements in the glands associated with the upper respiratory tract, Mueller et al., (1971) looked for plaque forming cells in these sites. After a single intravenous injection of sheep red cells no PFC were found in the Harderian gland, lacrimal gland, or caecal tonsil. However, PFC were found in the Harderian glands

after administration of the antigen directly on to the eye or into the eye orbit.

French et al., (1970) have shown that following the injection of a protein antigen with adjuvant intramuscularly, granulomata appear in which there are large infiltrations of plasma cells and germinal centres. Since a large amount of specific antibody can be extracted from these granulomata they suggest that this is a major site of production of antibody during the second phase of an adjuvant-antigen induced response.

Duffus and Allan, (1969) using an immunocytoadherence or "rosette" technique have demonstrated antibody producing cells of the plasmacyte series in the blood stream of chickens inoculated intramuscularly with heat-killed S.Gallinarum.

Attempts to demonstrate antibody production in the chicken's bursa of Fabricius have consistently met with failure. Jankovic and Mitrovic, (1967) and Glick and Whatley, (1967) using fluorescence techniques could not find anti-BSA producing cells in the bursa although the technique proved successful for detecting these cells in other sites (Jankovic and Mitrovik, 1967). Dent and Good, (1965) using the plaque technique also failed to find haemagglutinin or haemolysin producing cells in the bursa even after 3 intravenous injections of red cells. An early report by Kerstetter et al., (1962) of the production of anti-bovine γ -globulin antibodies by bursal

cells from pheasants given 2 or 3 injections of antigen may be unreliable since they did not show specificity of the fluorescent staining.

Although Jankovic and Mitrovik, (1967) report finding plasma cells, germinal centres and a small, but in their view significant, number of antibody producing cells in the chicken thymus, Dent and Good, (1965) are of the opinion that the numbers of such cells in the thymus are insignificant and might in fact be of haematogenous origin.

Taking all these reports into account then, it appears that antibody production can take place in any of the lymphoid tissues of the chicken except the bursa of Fabricius and perhaps the thymus, provided that the antigen reaches the tissues in great enough concentration and by a suitable route. Cellular immune reactions have been shown to be mounted by lymphocytes from blood, spleen and thymus but not from the bursa (Cain et al., 1968).

THE THYMUS AND THE BURSA OF FABRICIUS

The thymus and the bursa of Fabricius, therefore, are special cases. They are, in fact, the "central lymphoid organs" of the fowl, as distinct from the "peripheral" lymphoid tissues which have been described above as the site of cells actually participating in immune responses.

The concept of central and peripheral lymphoid organs was probably introduced by Fichtelius, (1960) who

suggested that the thymus provided cells which seeded to the spleen and there differentiated into cells capable of antibody production. The subject has since been elucidated through 3 major areas of research.

The Thymus: Extensive investigations by Miller and other groups of workers (Miller, 1961, 1962a, 1962b; Arnason et al., 1962; Jankovic et al., 1962; Waksman et al., 1962; Good et al., 1962; Miller and Osoba, 1963) established that neonatal thymectomy in mice (Miller, 1962a, 1962b; Good et al., 1962; Miller and Osoba, 1963), rats (Arnason et al., 1962; Jankovic et al., 1962; Waksman et al., 1962) and rabbits (Good et al., 1962) severely impairs their ability to mount cellular immune responses such as homograft rejection, and delayed hypersensitivity. It also greatly reduces their circulating pool of small lymphocytes and rather inconsistently reduces their capacity to produce serum antibodies to some antigens.

The way in which the thymus influences the development of immunological competence has been reviewed by Miller, (1965, 1966), Miller and Osoba, (1963) and Burnet, (1969).

The main facts are as follows:

1. Thymectomy has to be performed immediately after birth or else accompanied by near-lethal whole body irradiation to be effective in abolishing cellular immunity (Miller et al., 1963).
2. Complete recovery of immunological function can be brought about in lethally irradiated but non-

thymectomised mice by the administration of bone marrow cells even from neonatally thymectomised donors (Miller et al., 1963).

3. Repopulation of the lymphoid organs including the thymus in these lethally irradiated reconstituted animals has been shown by chromosome marking experiments to be effected exclusively by donor cells (Miller and Osoba, 1963).
4. Only bone marrow cells and not thymus, thoracic duct or lymph node cells can lodge and multiply in the thymus (Gowans and Knight, 1964).

Hence it has been postulated (Burnet, 1969; Miller, 1965) that lymphoid precursor cells originate in the bone marrow and migrate to the thymus where they differentiate under the influence of the radioresistant epithelial elements into immunologically competent cells. These subsequently seed to the peripheral tissues where they can multiply and function without further thymic influence.

This migration and differentiation is at its height during embryonic and neonatal life but stem cells do continue to differentiate and replenish the pool of competent cells during adult life.

The fact that immune competence has been shown to be restored to some extent by thymic grafts even when these are enclosed in Millipore chambers (Osoba and Miller, 1963) suggests that a humoral factor may be involved in the maturation process but does not preclude

the possibility that this factor normally acts inside the environment of the thymus (Miller, 1965).

The injection of non-cellular thymic extracts was shown by Metcalf, (1956) to produce a temporary lymphocytosis in the blood of new born mice, but Miller, (1963) failed to restore lymphocyte counts or homograft rejection in neonatally thymectomised mice with similar extracts. This, taken with the results of Millipore chamber grafting experiments, suggests that the epithelial-reticular structure of the thymus must be intact even for a hormonal maturation process to occur, (Osoba and Miller, 1963) or else that the restoration of competence by Millipore chamber grafts was due to a non-specific adjuvant effect as has been reported in experiments using bursa grafts in chickens (Dent et al., 1968).

The bursa of Fabricius: During the same period when the function of the thymus in mammals was being investigated, a great deal of interest had arisen as to the immunological function of the avian bursa of Fabricius. This was triggered off by the fortuitous discovery by Glick et al., (1956) that bursectomy prevented chickens from producing agglutinins to Salmonella typhimurium. The huge volume of literature on the effects of bursectomy which has been published since 1956 will be discussed in more detail in a later section. Only the main lines of evidence which have led to the concept of the bursa as a second central lymphoid organ will be described here.

From the beginning it was clear that bursectomy had to be carried out at an early age (not more than two weeks) to markedly affect antibody production (Chang et al., 1957; Mueller et al., 1960; Mueller et al., 1962). This paralleled the results of experiments on thymectomy in mice which showed that to inhibit immune competence thymectomy had to be carried out in the neonatal period (Miller et al., 1963).

There is, in fact, a notable similarity in ontogenic development and morphology between the bursa and the thymus. Both organs develop from the epithelium of the digestive tract and are lymphoepithelial in structure with lymphoid follicles organised into cortex and medulla (Ackerman and Knouff, 1964; Cain et al., 1968). Both develop independently of antigenic stimulation (Thorbecke et al., 1957), become lymphoid in nature earlier in embryogenesis than the other lymphoid organs (Papermaster and Good, 1962), and start to involute early in adult life.

The early experiments using neonatal surgical bursectomy (Chang et al., 1957) and more recent ones by Jankovic and Isakovic, (1967) and Arnason and Jankovic, (1967) showed a reduction rather than a total elimination of the ability to produce antibodies after antigenic stimulation. However more clear cut results were attained by the method of hormonal bursectomy introduced by Meyer et al., (1959) whereby the development of the bursa is prevented by an injection of 19-Nortestosterone

into 5 day old developing chick embryos, or when neonatal bursectomy was followed by whole body irradiation (Cooper et al., 1965). By these methods complete inhibition of antibody responses and even total agammaglobulinaemia could be produced in some individuals (Mueller et al., 1960; Aspinall and Meyer, 1964; Cooper et al., 1965; Warner et al., 1969). Bursectomised-irradiated birds also completely lacked evidence of germinal centre and plasma cell development (Cooper et al., 1965, 1966a).

Thus it was now clear that antibody production and immunoglobulin synthesis were totally dependent on the bursa of Fabricius, and that its role was not as had earlier been supposed (Jankovic and Isakovic, 1967) a secondary one.

Experiments to find out how the bursa exerts its influence on immunological development corresponding to the irradiation and reconstitution experiments on thymectomised mice have been few in chickens, largely because of the scarcity of syngeneic inbred strains. However, Moore and Owen, (1966) have used a sex chromosome marker to trace cellular migration during embryological development in chickens, and combining this technique with parabiosis of embryos of similar major histocompatibility types and transplantation of bursal rudiments onto the chorioallantoic membrane, have shown that, contrary to earlier opinion (Ackerman and Knouff, 1964), the majority of bursal lymphocytes are not derived from bursal epithelium, but are produced by proliferation

of blood borne stem cells. They suggest that the bursa provides a specific environment for the maturation of these cells into immune competent plasma cell precursors, and that testosterone treatment interferes with this environment.

Whether or not a hormone is involved in the maturation process is not clear. Several attempts have been made with inconclusive results to restore immune competence in bursectomised birds by saline bursal extracts (Glick, 1960; Mueller et al., 1964), grafts (Mueller et al., 1964; St. Pierre and Ackerman, 1965; Jankovic and Leskowitz, 1965; Dent et al., 1968), or cell suspensions (Cooper et al., 1966b). Mueller et al., (1964) reported a complete failure with both grafts and extracts, whereas other workers did achieve some enhancement of antibody production in bursectomised chickens by injecting saline bursal extracts (Glick, 1960) or by implanting bursal tissue enclosed in Millipore chambers (St. Pierre and Ackerman, 1965; Jankovic and Leskowitz, 1965; Dent et al., 1968). Dent et al., (1968), however, showed that the latter was a non-specific effect probably due to the adjuvant activity of contaminating bacteria in the chambers. Cooper et al., (1966b) were able to restore germinal centre formation, plasma cells and immunoglobulin synthesis in bursectomised irradiated chickens with bursal cell suspensions, but the birds were still unable to produce antibody to specific antigenic challenges.

Dissociation of Immunological Responsiveness: Even in those bursectomised chickens which are rendered completely agammaglobulinaemic, cellular immune competence is uninfluenced as measured by homograft rejection (Mueller et al., 1962; Warner et al., 1962; Aspinall and Meyer, 1964), graft versus host activity of blood leucocytes (Warner et al., 1962), delayed hypersensitivity to tuberculin, and the development of experimental allergic encephalomyelitis (Jankovic and Isvaneski, 1963). The unexpected finding by Warner et al., (1962) that bursectomy did inhibit delayed hypersensitivity reactions to tuberculin and vaccinia virus, has recently been reversed by these same workers (Warner et al., 1971).

On the other hand neonatal thymectomy in chickens, as in mice and rats, has been shown to abolish (Warner et al., 1962) or delay (Aspinall and Meyer, 1964) homograft rejection, whereas antibody responses are only partially depressed or may be uninfluenced, depending on the antigen (Marvanova and Hajek, 1969; Warner and Szenberg, 1964).

Through this research on avian immunology, then, it has become clear that immunological responses can be divided into two separate systems. On the one hand are the humoral immune responses mediated by antibodies produced by the plasma cell line which in the fowl is dependent for its development on the bursa of Fabricius, and on the other the cellular responses mediated by a

population of small lymphocytes dependent for their differentiation on the thymus.

This same dissociation clearly also occurs in mammals although the organ or tissues equivalent in function to the bursa have not yet been identified.

Evidence for the existence of a bursa equivalent in mammals is based on the fact that neonatal thymectomy in mice and rats prevents cellular immunity and reduces the peripheral small lymphocyte population, but does not prevent germinal centre or plasma cell development (Miller, 1962b; Jankovic et al., 1962; Waksman et al., 1962; Good et al., 1962a) and on observations on human patients suffering from various immune deficiency syndromes. The Bruton form of sex-linked recessive agammaglobulinaemia is almost identical to the deficiency produced by neonatal bursectomy plus irradiation (Cooper et al., 1965). These patients are deficient in plasma cells, and lack germinal centre organisation, whilst their cell-mediated responses and circulating lymphocytes are normal (Cooper et al., 1968). The Di George syndrome, on the other hand, mirrors the effects of neonatal thymectomy since germinal centres, plasma cells and immunoglobulin synthesis are normal, whilst the thymus is absent or poorly differentiated and cellular immunity and thymus dependent small lymphocytes are lacking.

Good and his co-workers have presented evidence including several extirpation experiments that the gut

associated lymphoid tissues are the homologue of the bursa of Fabricius in rabbits (Cooper et al., 1968; Perey et al., 1970b). Although the results have not been as clear cut as in the chicken system, and may not necessarily be extrapolated to other mammalian species, it does seem probable that these tissues have a bursa-like function in the rabbit.

Interaction of bursa and thymus dependent cells:

The thymus must also in some way influence humoral immune responses, since neonatal thymectomy decreases antibody responses to some antigens. How it does so is uncertain. The possible ways include:

1. a stem cell differentiation process in the thymus before differentiation occurs in the bursa or bursa equivalent (Cain et al., 1968);
2. a migration of cells from the bursa through the thymus (Woods and Linna, 1965; Cain et al., 1967);
3. a thymic hormone function; and
4. an interaction of thymus dependent "T" lymphocytes and bone marrow derived, bursa dependent "B" lymphocytes during the induction of an immune response.

This last possibility is the one which has gained most support and there is strong evidence in favour of such an interaction of cells. Germinal centres which have been shown to be bursa dependent (Cooper et al., 1965)

are usually located within or in close proximity to aggregates of thymus dependent small lymphocytes (Good et al., 1966).

Early studies by Taliaferro et al., (1952) and by Kohn, (1950) on the effect of irradiation at different stages during antibody responses, provided evidence of an initial radiosensitive phase followed by a radio-resistant phase. This could be interpreted as being due to the involvement of two different cell populations. More direct evidence of an interaction between "T" and "B" lymphocytes was provided by experiments by Claman et al., (1966). They showed that in heavily irradiated mice the inoculation of a mixture of bone marrow and thymus cells allowed the production of more haemolysins against sheep red cells than could be accounted for by summing the activities of each cell population alone. In a similar type of experiment Mitchell and Miller, (1968) showed that "T" lymphocytes from the thymus or thoracic duct could increase the number of haemolysin forming cells in the spleen of neonatally thymectomised mice inoculated with sheep red cells. They further provided evidence that it was the recipients' own "B" cells which were the precursors of the antibody forming cells and that the "T" cells had first to react with specific antigen before interaction with the "B" cells would produce a significant haemolysin response. Hence they postulated that "T" lymphocytes recognise

antigen and interact with it in some way that triggers off the differentiation of "B" lymphocytes to antibody producing cells.

The interaction is not an essential part of antibody production for certain antigens which are known to be "thymus independent". Møller, (1970) showed that soluble serum protein antigens were more "thymus dependent" than sheep red cell antigens and that E.coli lipopolysaccharide was completely "thymus independent" since the thymus cells not only did not add to, but appeared to detract from the antibody response to this antigen. Similar findings have been reported by Humphrey et al., (1964) for Pneumococcus polysaccharide. Møller, (1970) has suggested that the "T" lymphocytes may act merely as an antigen concentrating mechanism, holding the antigen in close contact with the "B" cell receptors. Macrophages could act in the same way, and the repeating antigenic determinants on polysaccharide molecules would allow binding to multiple receptors, thus making dissociation unlikely and a helper cell unnecessary. Other possible mechanisms for the cell interaction have been suggested, such as the transfer of an informational message via RNA, or the release of either non-specific lymphokine factors or an antigen specific factor by stimulated "T" cells. However, in the absence of any decisive evidence, the question must remain open.

OBJECTIVES

Current immunological research, then, is very much concerned with the bursa and thymus and their dependent cell populations.

It is obvious that, because of its positively identified separate central lymphoid organs, the chicken offers a unique model for this research. The bursectomised, agammaglobulinaemic chicken, is the only experimental system in which the thymus dependent cells can be studied in the absence of the antibody producing system and its circulating products, the serum immunoglobulins.

The chicken's obvious value as an experimental animal is, however, detracted from by the inadequacy of present knowledge on avian immune responses and in particular on the immunoglobulins.

The aim of the work described in this thesis, was to contribute to present knowledge on immunoglobulins in this species and to use the experimental model of the bursectomised chicken to study certain aspects of immunoglobulin synthesis and function.

This was approached by the following series of studies:

1. further characterisation studies on chicken immunoglobulins;
2. a quantitative investigation of the synthesis of these proteins at various stages during ontogenic development, and the effect on this of surgical

- bursectomy at different times during embryonic life;
3. a study of antibody production and resistance to infection in relation to immunoglobulin synthesis in bursectomised birds;
 4. the demonstration of the presence of immunoglobulin determinants on the surface of chicken lymphocytes, and studies to determine the proportions of determinant-bearing lymphocytes in the thymus dependent and bursa dependent systems.

SECTION II

IMMUNOGLOBULIN STUDIES

INTRODUCTION

From the general introduction it will be realised that the literature on chicken immunoglobulins is far from comprehensive. Whilst it seems generally accepted that avian IgM antibodies correspond in most details with mammalian IgM, the reports on the properties of 7S antibodies have in many cases been contradictory and suggest that different authors might actually be describing more than one type of 7S immunoglobulin (Orlans, 1968).

Moreover, most of the work has been carried out on specific antibodies rather than normal serum immunoglobulins, and few reports on the latter are available. Those which have been published stress the dissimilarities between chicken 7S γ -globulins and mammalian IgG (Tenenhouse and Deutsch, 1966; Benedict, 1967; Leslie and Clem, 1969). These differences include a higher electrophoretic mobility, a lower isoelectric point, a higher sedimentation coefficient, a higher hexose content, and different dissociation properties. Unlike human or rabbit

γ -globulins, those of the chicken can be split by pepsin in the absence of a reducing agent, and reduced and alkylated chicken IgG partially dissociates to heavy and light chains at pH 7.2-8.2 without a dispersing agent, suggesting that the noncovalent bonds in the molecule are weak. Chicken IgM resembles IgG in this

respect, thus also differing from its mammalian counterpart. Leslie and Clem, (1969) have suggested that chicken 7S γ -globulins should be called IgY since they do not closely resemble any of the known mammalian immunoglobulin classes. Tenenhouse and Deutsch, (1966), on the other hand, have likened them to IgA.

In view of these discrepancies in the literature on the properties of chicken immunoglobulins, and the variation in methods reported for their purification (Szenberg et al., 1965; Tenenhouse and Deutsch, 1966; Benedict, 1967; Orlans, 1968; Gallagher and Voss, 1969; Leslie and Clem, 1969), it was considered that further characterisation studies would be valuable.

It was decided to concentrate on two immunoglobulins for these studies; the one which resembles mammalian IgG in its immunoelectrophoresis appearance, and the more closely analogous IgM. Modern serological methods based on the antigenic properties of the immunoglobulins would be employed for quantitative measurements rather than those based on antibody titration, since in this way it would be certain that the same 7S immunoglobulin was being studied throughout the programme of work. It was hoped that the results could then be related to the findings of other workers.

In addition to the IgG and IgM an attempt has also been made to isolate other chicken immunoglobulin classes.

MATERIALS AND METHODS

Immunoelectrophoresis: This was carried out by a method essentially similar to that of Grabar and Williams, (1953). 1% Difco Noble agar in 0.05 ionic strength (μ) barbitone acetate buffer was used on 10 x 10 cm. or 5 x 10 cm. glass slides, with 0.025 μ barbitone acetate buffer in the electrophoresis bath. The current and duration of run were varied according to requirements. The microtechnique of Scheidegger, (1955) was also used for some samples. In this case immunodiffusion agar (Oxoid 1D agar) at 0.9% in 0.05 μ barbitone acetate buffer, or Ion agar No. 2 (Oxoid) at 1% in 0.025 μ buffer were used, with 0.025 μ buffer in the bath.

For preservation the slides were soaked for 24 hours in saline, rinsed in distilled water, overlaid with moist filter paper and dried overnight in an incubator at 37°C. They were then fixed for 5 minutes in 2% aqueous acetic acid, stained with 0.5% amido black in methanol/glacial acetic acid 9 : 1 for 7 minutes, and decolourised with methanol/glacial acetic acid 9 : 1 for 15 minutes.

Single Radial Immunodiffusion (SRD): A modification of the method described by Mancini et al., (1965) was used. Preliminary tests determined the optimum dilutions of anti-IgG, -IgM or -7S γ 1 antisera for incorporation into the agar. For these microscope slides were covered with 2.5 ml. volumes of agar prepared as follows: 1.25 ml. volumes of melted 2% Difco Noble agar in SRD buffer

(0.03M K_2HPO_4 + 0.1M NaCl, pH 8.2) were added to equal volumes of 1/5, 1/10, 1/15 and 1/20 dilutions of ^{anti} Λ -IgG, -IgM and -7S γ 1 in the same buffer, mixed, and immediately poured onto the slides. Four wells were made in each slide and filled with normal chicken serum diluted 1/2, 1/4, 1/8 and 1/16. Diffusion was allowed to continue for 18 hours at 5°C after which the slides were examined. The optimum dilutions of the antisera were taken as the highest dilution which gave clearly defined rings of precipitation - these were 1/20 for anti-IgG and anti-IgM and 1/10 for anti-7S γ 1.

For quantitative estimations on experimental samples 10 x 10 cm. glass plates were used, which held 16-20 ml. agar. A template was used to cut out 36 wells, (Fig. 1), the plugs of agar being removed by suction. The first 6 wells were used for dilutions of a standard pooled adult serum sample, leaving 30 for test samples. Care was taken to ensure that all wells were filled to the brim to give uniformity of sample volume. Very finely drawn pasteur pipettes were used for this purpose. The plates were kept in a moist chamber at 5°C for 18 hours until the precipitin rings appeared. A typical plate is shown in Fig. 2. If the rings were well defined their diameters were measured while the slide was wet. For the measurement of fainter slides the plates were first dried and stained by the method described previously for immunoelectrophoresis. Using a magnifying lens incorporating a micrometer scale, 5 different diameters

Figure 1. S.R.D. Analysis: Plate holder with template above and gel cutter in situ.

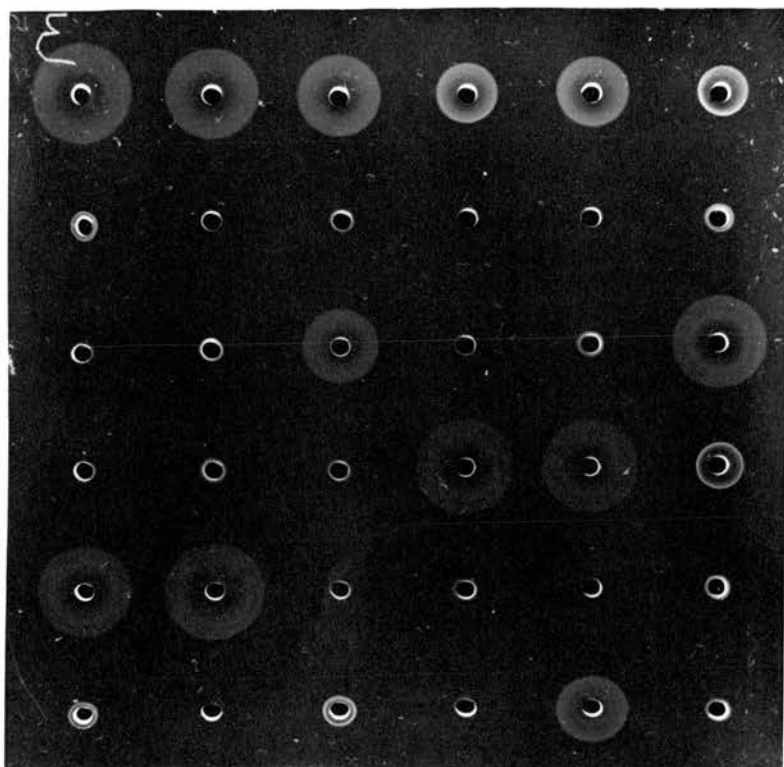
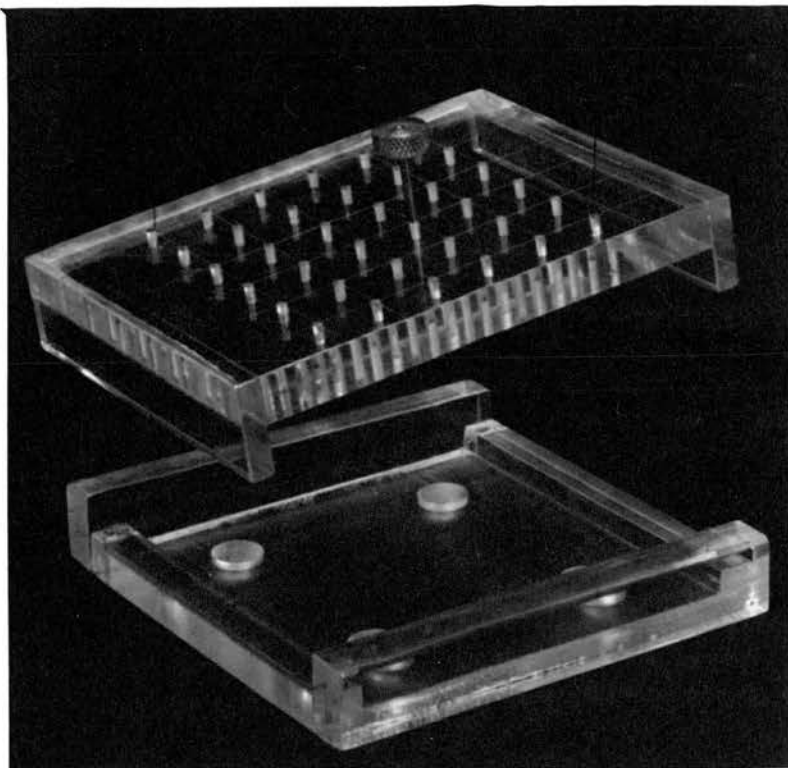


Figure 2. S.R.D. Analysis: A typical plate with 30 individual chicken sera. The top row contains the reference serum titration.

of each ring were measured, and an average taken. The ring diameters of the standard serum dilutions were plotted on semi-logarithmic graph paper to prepare a calibration curve. This gave a linear relationship from which the sample ring diameter readings could be converted to percentages of the standard. All IgG, IgM and 7S γ 1 values are therefore expressed as a percentage of the amount of that protein contained in the standard pooled adult serum sample.

In accordance with the findings of Fahey and McKelvey, (1965) the error in this method of measurement of serum proteins was found to be $\pm 10\%$.

Double Diffusion Precipitation (Ouchterlony, 1953):

This was carried out using 1% Difco Noble agar in SRD buffer on microscope slides.

Sephadex G-200 Gel Filtration: 2 ml. normal chicken serum was dialysed for 24 hours against 0.1M Tris HCl buffer pH8 and fractionated on a 600 x 30 cm. column of Sephadex G-200 (Pharmacia Ltd., London) using this elution buffer. The eluate was collected by a timed operation in approximately 10 ml. volumes. Each tube was treated separately and was concentrated 5 X by dialysis in polyethylene glycol (Carbowax 6000, Union Carbide Co.), dialysed for 24 hours at 4°C against distilled water, and freeze-dried. Each freeze-dried fraction was then weighed and redissolved in the appropriate volume of 0.15M NaCl to give a concentration of 10 mg./ml.

Quantitative measurements of the immunoglobulin contents of the fractions were then carried out by single radial immunodiffusion as already described. The quantity of each protein in each fraction was expressed as a percentage of the total amount of that protein eluted from the column.

DEAE Cellulose Chromatography: 5 ml. chicken serum (a hyperimmune anti-S.gallinarum serum) was fractionated on a 24 x 30 cm. column containing DEAE cellulose (Whatman DE 32), using stepwise elution with a series of sodium phosphate buffers. The serum was first equilibrated against the starting buffer by dialysis. Some precipitation occurred at this stage, but since it was not a large amount the sample was cleared by centrifugation and the fractionation continued.

The series of buffers was as follows:

- | | |
|-------------------------------------|----------------|
| 1. 0.01M phosphate pH7.5 | 2. 0.03M pH7.3 |
| 3. 0.05M pH7.0 | 4. 0.09M pH6.7 |
| 5. 0.1 M pH6.4 | 6. 0.2 M pH5.8 |
| 7. 0.3 M pH5.4 | 8. 0.4 M pH4.7 |
| 9. 0.4 M phosphate + 2M NaCl pH4.4. | |

10 ml. fractions were collected and treated as already described for sephadex G-200 fractions.

Radioimmuno-electrophoresis: Serum and tears from chickens stimulated with BSA were examined for antibody activity by the radioimmuno-electrophoresis technique described by Bloch et al., (1968).

Immuno-electrophoresis was carried out with chicken

serum or tears in the wells and anti-whole serum or anti-7S γ 1 in the troughs. After the precipitin arcs had appeared the slides were washed for 48 hours with several changes of saline. ^{125}I labelled BSA was then added to the troughs and the slides left for 24 hours at 4°C . At the end of this period they were washed for 2 days with several changes of saline, and for a further 2 days with distilled water. They were then dried and overlaid with X-ray film. After 72 hours the films were developed. The slides were then stained as normal with amido black.

Indirect Haemagglutination and Antiglobulin

Haemagglutination

Reagents:

1. Hyperimmune chicken anti-S.gallinarum antiserum, complement inactivated for 30 minutes at 56°C , and absorbed twice with 6 X washed fowl and bovine red blood cells; or heat inactivated fractions of chicken anti-S.gallinarum antiserum eluted from DEAE cellulose.
2. 1.5% suspensions of 6 X washed fowl or bovine red cells sensitised with S.gallinarum polysaccharide. The polysaccharide was prepared by the phenol-water method of Westphal as modified by Ravin et al., (1960), freeze-dried, and reconstituted in sterile 0.85% NaCl at a concentration of 5 mg./ml. $\frac{1}{2}$ ml. of this solution was added to 2 ml. 0.02N NaOH and incubated for 2 hours at 37°C , after which

the pH was brought back to 7.2 by the slow addition of 0.1N HCl using 2 drops of bromothymol blue as indicator. At the optimum pH the solution turned green. The alkaline treatment greatly increases the sensitising ability of the polysaccharide (Neter et al., 1956). The red cell suspensions were sensitised by the addition of 0.2 ml. polysaccharide to 10 ml. of a 1.5% suspension, and incubation for 1 hour at 37°C. The free antigen was then removed by washing the cells 3 X in saline.

3. Anti-IgG, -IgM or -7S γ 1 antisera, heat inactivated at 56°C for 30 minutes and absorbed twice with 6X washed fowl and bovine erythrocytes.
4. Sheep anti-rabbit IgG antiserum. This was prepared in the following way: the globulins from 10 ml. normal rabbit serum were precipitated with 18% Na_2SO_4 and redissolved in 5 ml. normal saline. After dialysis against 0.01M phosphate buffer pH7.5 the globulins were fractionated on a 2.4 x 30 cm. DEAE cellulose column. The protein peak eluted with the 0.01M phosphate starting buffer was concentrated by ultrafiltration, freeze-dried, then tested by immunoelectrophoresis with anti-rabbit whole serum and found to be pure IgG (Fig. 3a). It was, therefore, dissolved in sterile saline at a concentration of 10 mg./ml. and emulsified in Freund's incomplete adjuvant. 9 ml. of the emulsion was

injected subcutaneously into a sheep at various sites over the animal's back. This was repeated 2 weeks later and in a further 2 weeks 2 ml. IgG was injected intravenously. 6 days later the sheep was bled and its serum tested by immunoelectrophoresis against normal rabbit serum. Only an IgG line appeared (Fig. 3b). The antiserum was heat inactivated and absorbed with normal chicken serum and washed bovine red cells.

Indirect Haemagglutination test: 0.1 ml. volumes of 1% modified erythrocytes were added to equal volumes of twofold dilutions of serum starting from 1/2 and mixed. The haemagglutination titre was determined after incubation at 37°C for 30 minutes or until the red cells settled.

Antiglobulin (Coombs) test: The erythrocytes in the serum dilutions showing no haemagglutination were washed 3 times in physiological saline and each sample finally resuspended in 0.2 ml. of saline. 1 drop (0.02 mls.) of erythrocyte-absorbed antiglobulin serum was added to each tube. The tubes were then shaken and the antiglobulin titre was determined after a further incubation at 37°C.

Controls:

1. Modified cells in saline.
2. Unmodified cells with 1/2 antiserum.

Figure 3a.

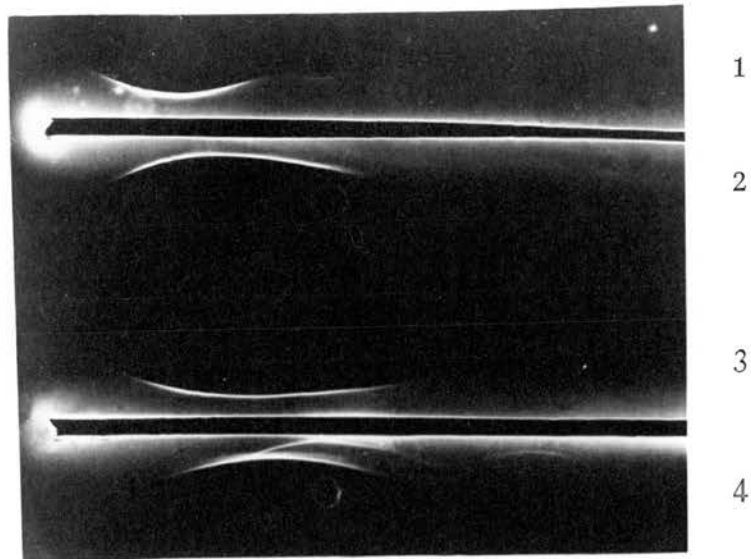


Figure 3b.

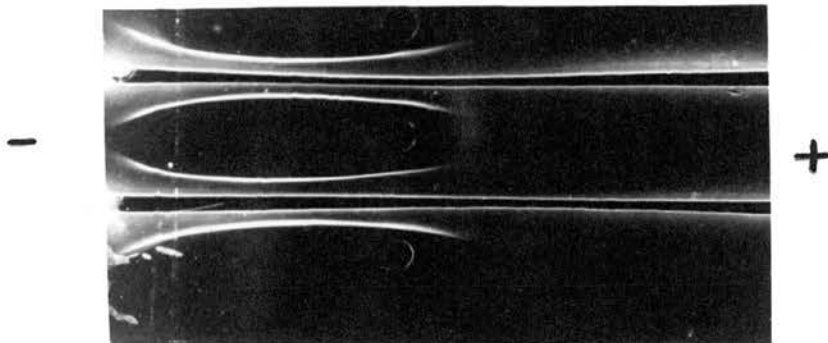


Figure 3a. Immunoelectrophoretic analysis of DEAE cellulose fractionation of normal rabbit serum.

Wells 1. Peak I 0.01 M phosphate (pure IgG)
2. Peak II
3. Peak III
4. Peak IV

Troughs both contain anti-whole rabbit serum.

Figure 3b. Immunoelectrophoretic analysis of sheep anti-rabbit IgG serum. Wells contain normal rabbit serum. Troughs contain the anti-IgG antiserum.

EXPERIMENTAL STUDY

1. Preparation of Specific Anti-immunoglobulin Sera:

The initial problem was to produce specific anti-immunoglobulin antisera which could then be used for quantitative measurement of the immunoglobulins.

An anti-whole serum antiserum was raised first in rabbits as follows:

a. Rabbit anti-chicken whole serum: Adult New Zealand white rabbits were injected intramuscularly with 2 ml. of an emulsion of undiluted chicken serum in Freund's complete adjuvant, 1 ml. into each hind leg. This procedure was repeated 2 weeks later and in a further 2 weeks was followed by an intraperitoneal injection of 1 ml. of a 1/10 dilution of chicken serum in sterile 0.85% NaCl. Thereafter a series of 5 intravenous injections were given, one every second day, in doses increasing from 0.1 to 1 ml. of a 1/10 dilution of serum. The rabbits were bled from the ear vein 7 days after the last inoculation, and the serum separated and stored at -20°C until use.

b. Rabbit anti-chicken IgG: The intention was then to use the method of Goudie et al., (1966) to prepare specific anti-IgG and anti-IgM antisera. This involved the initial preparation of a polyvalent rabbit anti-immunoglobulin serum with which to obtain IgG and IgM immunoelectrophoresis

precipitin lines, which could then be used as antigen for specific antiserum production. For this purpose 4 White Leghorn pullets were each given a single intravenous injection of 40 mg. bovine serum albumin (BSA) and bled 9 days later. The serum was separated and pooled. Equal volumes of this undiluted antiserum were added to two-fold doubling dilutions of 5% BSA to find the optimum dilution of antigen for maximum precipitation of antibody. A 1/64 dilution of 5% BSA was judged to be the optimum by visual examination for opacity, and so 16 ml. of this dilution of BSA in 7% NaCl was added to an equal volume of the chicken anti-BSA antiserum and precipitation allowed to continue for 24 hours at 4°C. The precipitate was then recovered by centrifugation, washed 6 times in 7% NaCl and weighed. It was made up to a 10% solution in sterile 0.85% saline and used to inoculate one New Zealand white rabbit (B.112) following the same inoculation schedule as previously described. Seven days after the last injection a small volume of blood was taken and the serum tested by immunoelectrophoresis using whole chicken serum in the wells. It was anticipated that this antiserum would have activity against chicken IgG and IgM. In fact only one line appeared in the γ region and this was identified as IgG on the basis of its similar appearance to mammalian IgG (Fig. 4). Instead therefore of continuing with the method which involves cutting out the precipitin arcs and

injecting these into individual rabbits, a large volume of antiserum was taken from rabbit B.112 and stored at -20°C for use as anti-IgG antiserum.

c. Rabbit anti-chicken IgM: As has been found by many workers considerable difficulty was experienced in the preparation of a specific anti-chicken IgM serum. As already mentioned the first method proved unsuccessful for this purpose. Consequently several other methods for preparing pure IgM antigen for rabbit inoculation were tried. Briefly, these included: (i) fractionation of chicken serum by gel filtration through Sephadex G-200, followed by immunoelectrophoresis of the first (19S) peak material using anti-whole chicken serum in the troughs, excision of the arcs identified as IgM, and treatment by the method of Goudie et al., (1966) for rabbit inoculation; (ii) dialysis of chicken serum for 24 hours at 4°C against distilled water. (With mammalian sera this produces an IgM rich euglobulin precipitate (Penhale et al., 1971).) The precipitate was then washed 4 times in distilled water, redissolved in borate buffered saline and filtered through Sephadex G-200. The first peak material was freeze-dried and used for inoculation; (iii) preparation of immune complexes by agglutination of 4 x washed S.gallinarum bacterial cells with a peak I Sephadex G-200 fraction of chicken anti-S.gallinarum antiserum. The agglutinated cells were washed 6 times in phosphate buffered saline and resuspended in sterile saline for inoculation.

Both rabbits and guinea-pigs were used for inoculation with antigen prepared by these methods but at no time was a specific anti-IgM serum obtained. In most instances, in fact, when the antisera were tested by immunoelectrophoresis with whole chicken serum, only a line identified as IgG appeared.

Finally it was decided to raise a polyvalent anti- γ -globulin serum and render it specific by absorptions.

The following method proved successful and has been adopted exclusively: Precipitation of globulins from normal chicken serum was carried out by dialysis against 24% Na_2SO_4 and also by the method described by Benedict, (1967). The precipitate from 30 ml. chicken serum was dissolved in 6 ml. borate buffered saline and dialysed against this buffer for 72 hours. After cleaning by centrifugation, the globulin solution was fractionated by gel filtration through a 600 x 30 cm. column of Sephadex G-200 using the borate buffer. The first peak fractions were pooled, concentrated by ultrafiltration, and recycled through Sephadex G-200. The first peak was again collected, concentrated, dialysed against distilled water, and freeze-dried. It was then resuspended in sterile 0.85% saline and used for inoculating 4 adult rabbits. Following gel filtration it was hoped that this preparation would contain mainly IgM and thus stimulate strong anti-IgM activity in the antisera. The initial inoculation schedule was as already described, but it was found that 7 additional

intravenous injections were necessary before a strong IgM line appeared on immunoelectrophoresis. At this stage the antisera showed activity against several γ components (Fig. 5a). Complete specificity for μ chain determinants was obtained by absorption with serum from newly hatched chicks which contains IgG transmitted from the hen via the yolk but no IgM. When the absorbed sera were tested by immunoelectrophoresis and double diffusion in agar gel with whole chicken serum, only one line appeared, in position and shape characteristic of IgM (Fig. 5b).

d. In addition to the IgG and IgM an attempt was made to isolate and study other chicken immunoglobulins. One way in which this problem was approached was to prepare a specific antiserum against a serum protein shown by immunoelectrophoresis to have a γ_1 mobility. This antiserum was then used for characterisation studies and in experiments to ascertain whether or not the protein was in fact an immunoglobulin.

2. Rabbit anti-7S γ_1 protein: This antiserum (called anti-7S γ_1 because the protein appeared in the 7S peak in subsequent gel filtration studies) was prepared by the method reported by Goudie et al., (1966) using as antigen an immunoelectrophoresis precipitin arc. Gamma globulins were first precipitated from a secondary response chicken anti-BSA serum by dialysis against 15% sodium sulphate. This precipitate was then dissolved in a small volume of NaCl and subjected to immunoelectrophoresis against anti-

Figure 4. Immuno-electrophoresis slide showing specificity of rabbit B112 anti-chicken IgG antiserum. Wells contain whole chicken serum. Trough contains rabbit B112 anti-chicken IgG.

Figure 5. Immuno-electrophoresis slides showing:
a) polyvalent anti- γ globulin serum;
b) the same antiserum after absorption with day old chicken serum, showing residual activity only against IgM.
Both wells contain whole chicken serum.

Figure 6. Immuno-electrophoresis slides showing:
a) 15% sodium sulphate precipitated chicken γ globulins, with anti-whole chicken serum in the trough. The precipitin line used for rabbit inoculation is indicated by arrow.
b) rabbit anti-7S γ 1 antiserum prepared by injecting the precipitin arc shown in Fig. 6a into rabbits. The wells contain whole chicken serum.

Figure 4.

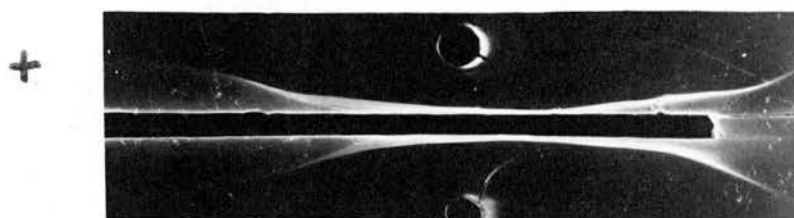


Figure 5 a b

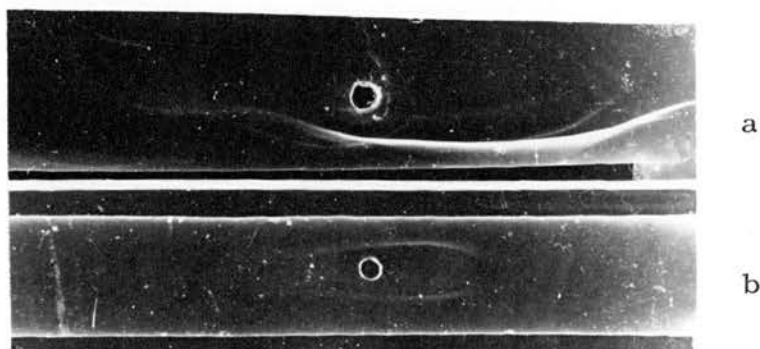
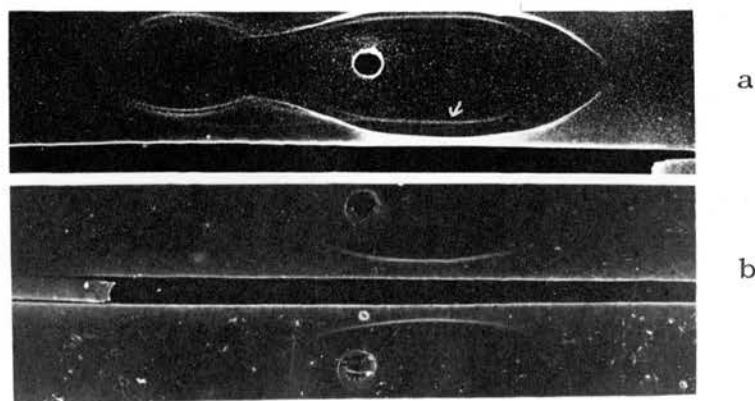


Figure 6 a b



chicken whole serum (Fig. 6a). Precipitin arcs in the $\gamma 1$ region which did not resemble either IgG or IgM were excised and used for rabbit inoculation. When the resulting rabbit antiserum was tested by immunoelectrophoresis against normal chicken serum it gave a single line as shown in Fig. 6b. Subsequent antiserum preparations obtained by restimulation of the rabbit with precipitin arcs produced by the reaction of the first preparation of antiserum with normal chicken serum showed activity against IgG as well as the 7S $\gamma 1$ protein. The three specific antisera were found to give good results when used for SRD analyses as described in the Materials and Methods section.

2. Characterisation of IgG, IgM, 7S $\gamma 1$:

The antisera were then used in a detailed quantitative study of the elution characteristics of these proteins from Sephadex G-200 and DEAE cellulose.

The details of these fractionation procedures have been described in Materials and Methods.

Fig. 7 shows the elution profile of normal chicken serum obtained by Sephadex G-200 gel filtration.

As expected, the greater proportion of the protein identified antigenically as IgM came through in the void volume but a considerable amount was also recovered in the second 7S peak. IgG started to

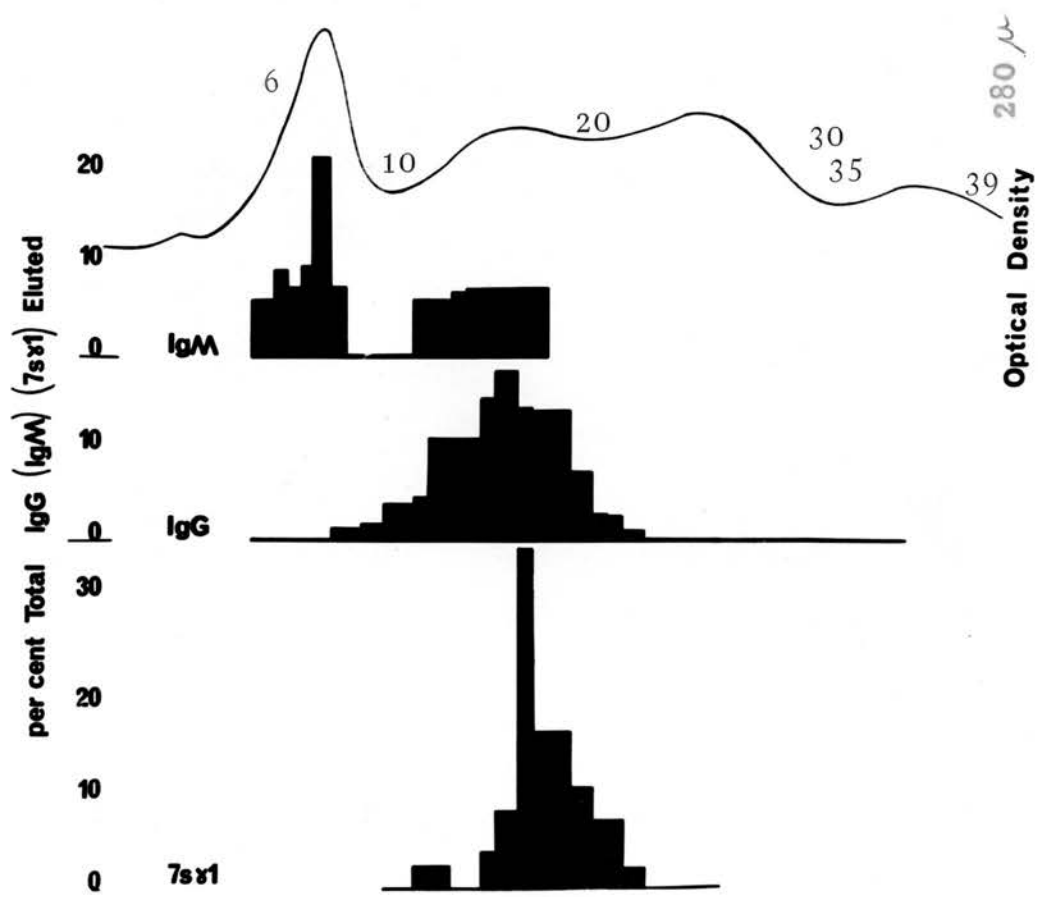


Figure 7 **Sephadex G 200 Elution Profile
For Chicken Immunoglobulins**

appear towards the end of the 19S peak and reached its highest concentration in the middle of the 7S peak.

Very little extended into the third peak fractions. The

γ 1 protein was also found in the 7S peak. It started to elute slightly later than the IgG and was confined to a smaller range of tubes, but reached its maximum concentration at about the same time as IgG.

The chromatographic separation of IgG, IgM, and 7S γ 1 on DEAE cellulose is shown in Fig. 8. The serum used in this instance was a hyperimmune anti-S.gallinarum anti-serum.

The fractions eluted with buffers 1, 2 and 3 contained only IgG, although peak 2 had very little, and peak 3 had only trace amounts of this immunoglobulin. Peak 4 contained a large percentage of the IgG, almost all the 7S γ 1 and a small amount of IgM. Peak 5 contained most of the remaining IgG and a small quantity of the 7S γ 1. The great bulk of the IgM appeared in peaks 6 and 7, with only trace amounts of the other proteins. Peak 8 had traces of IgM and IgG, but most of the protein eluted with buffer 9 was not identified.

Discussion

The results obtained from Sephadex G-200 fractionation can be compared with reports in the literature on the use of this method for the purification of chicken IgG and IgM (Benedict, 1967; Leslie and Clem, 1969) and for the separation of primary and secondary response antibodies

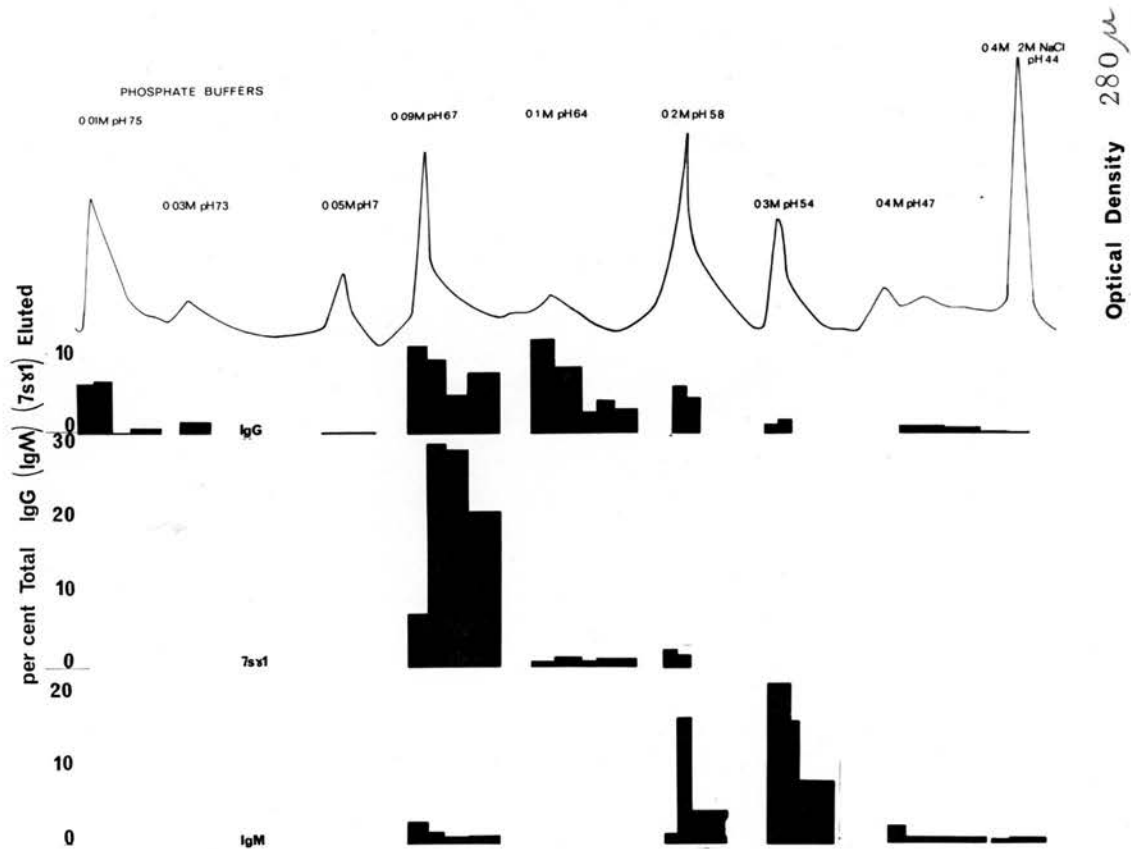


Figure 8

DEAE Cellulose Elution Profile
For Chicken Immunoglobulins

(Riha, 1965; Dreesman et al., 1965). Benedict, (1967) suggests that pure IgM can be obtained from first peak material by recycling through Sephadex G-200, whilst the second peak yields IgG which is essentially free of IgM but is contaminated with a β -globulin. Riha, (1965) and Dreesman et al., (1965) found that primary response haemagglutinating antibodies were almost exclusively confined to the first Sephadex G-200 peak, whilst during secondary responses haemagglutinating activity appeared in both peaks. Most of the precipitating antibody, on the other hand, was in the 7S fraction, during both primary and secondary responses. Radioimmuno-electrophoresis of the two peaks revealed antibody activity in the IgG arc in second peak material and in the IgM arc in first peak material.

As previously mentioned, Orlans, (1968) has suggested that the second Sephadex G-200 peak contains more than one type of immunoglobulin. The suggestion is based on her own finding of two different 7S antibodies produced in response to DNP, and on the observation that other workers had reported remarkable variations in the properties of chicken 7S γ -globulins (Tenenhouse and Deutsch, 1966; Dreesman and Benedict, 1965a; Benedict et al., 1963; Rosenquist and Campbell, 1966).

The present fractionation study has shown a 7S γ -globulin antigenically distinct from IgG in the second peak. It has also shown, as others have, that the first peak is

almost exclusively IgM, but in addition a considerable amount of 7S IgM was recovered in the second peak along with the IgG. Whether or not the 7S γ 1 identified here bears any relation to the 7S antibodies described by other workers cannot be determined at this stage. The finding of a 7S type of IgM is not unique but has been reported for shark serum (Clem and Small, 1967) and human cord serum (Perchalski et al., 1968). The 7S IgM might alternatively represent subunits produced by breakdown of some normal 19S IgM molecules. It is not known whether this particular molecular species of IgM has antibody activity.

DEAE cellulose chromatography has been used by a number of workers for the separation of chicken immunoglobulins and antibodies (Dreesman et al., 1965; Leslie and Clem, 1969; Gallagher and Voss, 1969; Szenberg et al., 1965; Benedict et al., 1963; Benedict, 1967), mainly as a last stage in the purification process. Most of these reports are not comparable with the present results since different buffer systems were used. However the last 5 buffers used in this study were used by Dreesman et al. for separating anti-BSA antibodies, and by Benedict, (1967) for purification of IgG and IgM, and, taking into consideration the fact that their first buffer (0.1 M Phosphate pH 6.4) would elute all the proteins eluted here separately with buffers 1, 2, 3, 4 and 5, the results are essentially similar. One point of difference is their finding of IgM



antibodies mainly in the 0.3 and 0.4 M fractions, whereas here most of the IgM was eluted with the 0.2 and 0.3 M buffers. Also of interest is the fact that these workers eluted 3 to 5 times more globulin with the 0.1 M buffer than with the 0.2 M buffer and yet the antibody activity of each fraction was about the same. The bulk of the globulin in their first peak may have been the 7S γ 1 protein described here. In earlier experiments the same group of workers had used a buffer system starting with 0.0175 M Phosphate pH 6.3 but had found that a great deal of globulin precipitated in that buffer and that separation of antibodies was poor. This has not been the case in the present study, where separation of IgG and IgM was good, and only a small amount of precipitation occurred during dialysis with the starting buffer.

It was not possible with either method of fractionation to obtain 7S γ 1 free of IgG. Pure IgG was obtained by DEAE cellulose chromatography, and IgM relatively free of both IgG and 7S γ 1 by both methods. Each separate fraction from both columns was also analysed by immunoelectrophoresis using anti whole chicken serum in the troughs. Electrophoretic studies are shown in Figs. 9 - 13. Although these provide useful reference slides for future immunoelectrophoretic studies of chicken secretions etc. they yield very little positive information. The great variability in the appearance of the immunoglobulin arcs with different concentrations of the reagents

Figure 9. Immuno-electrophoretic analysis of chicken serum fractions eluted from Sephadex G-200. Fractions 6 to 10 (first 19S peak). Fractions 11 to 20 (second 7S peak). All troughs contain anti-whole chicken serum.

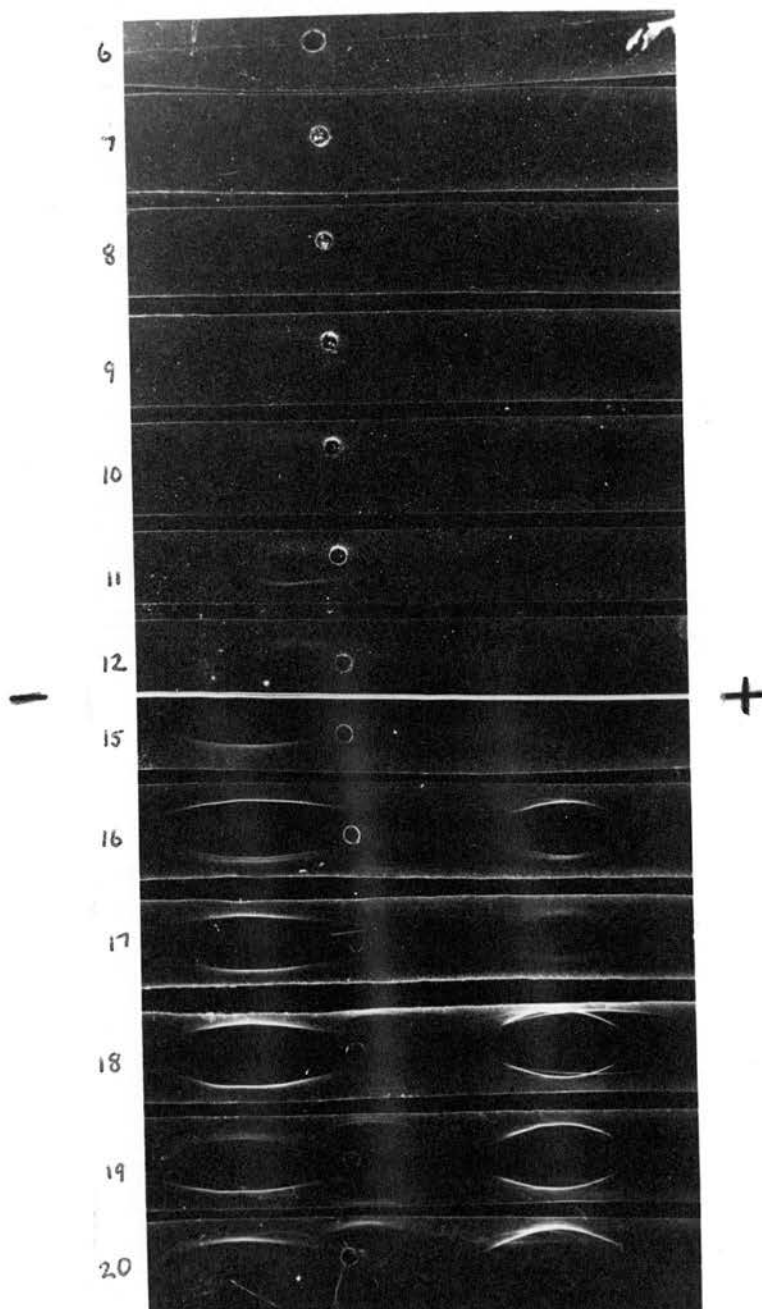


Figure 10. Immuno-electrophoretic analysis of Sephadex G-200 fractionation of normal chicken serum.

Fractions 21 - 33 (third albumin peak).

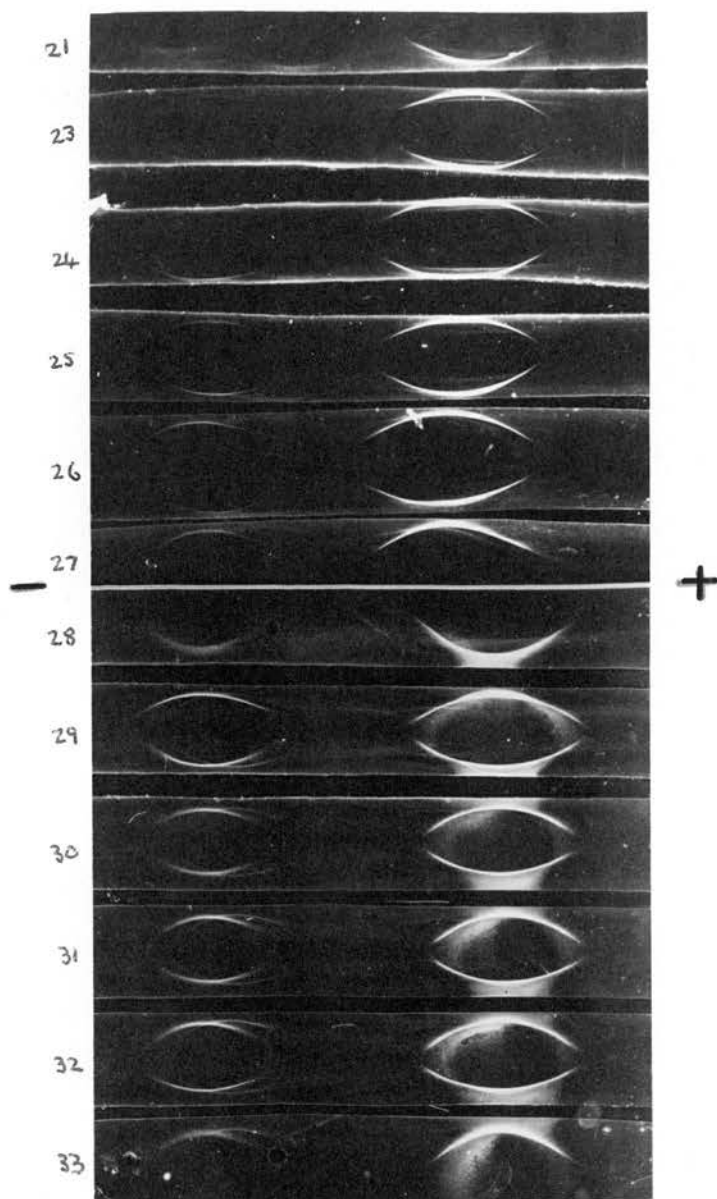


Figure 11. Immuno-electrophoretic analysis of chicken serum fractions eluted from DEAE cellulose.

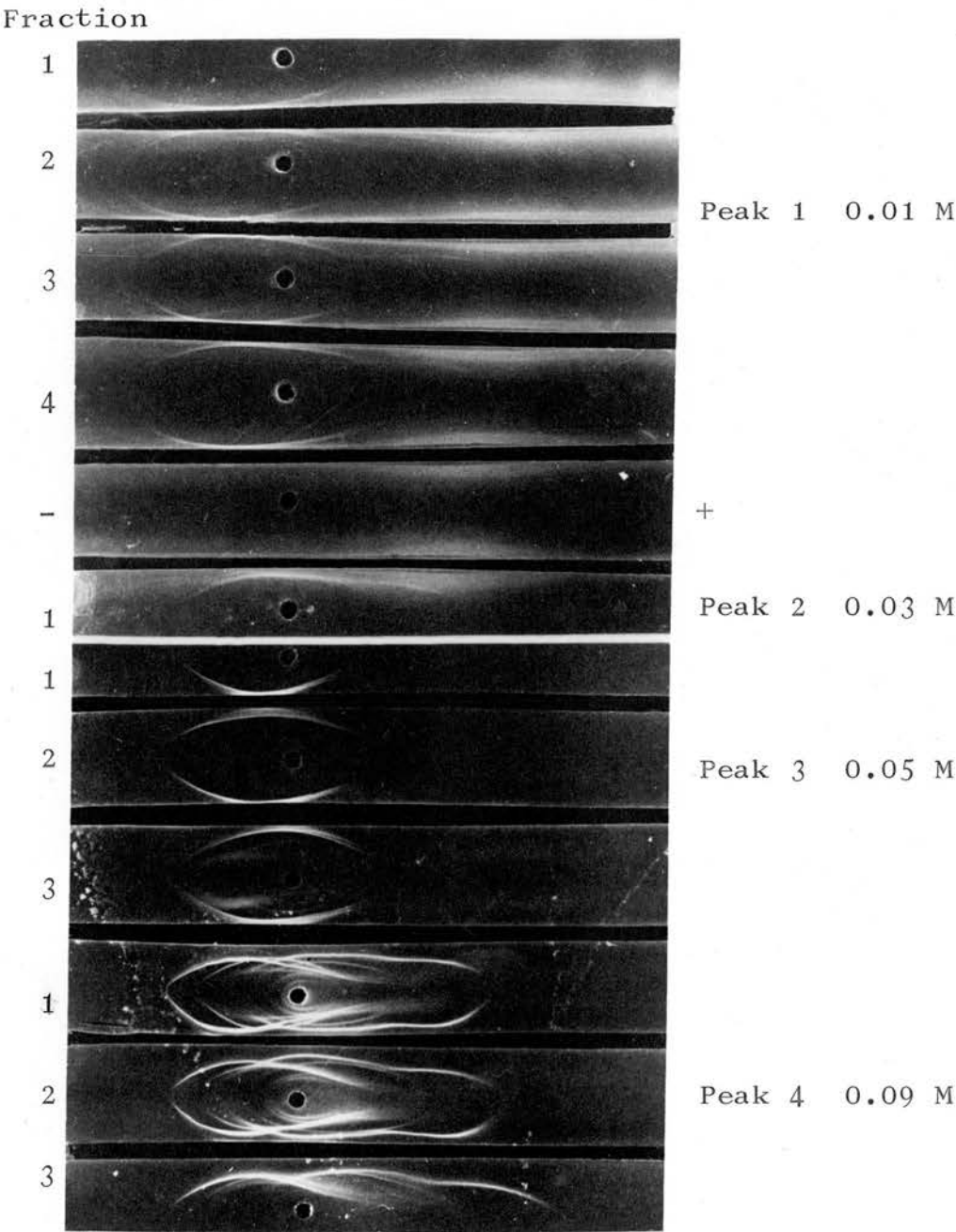


Figure 12. Immuno-electrophoretic analysis of chicken serum fractions eluted from DEAE cellulose.

Fraction

4

Peak 4 0.1 M

1

2

Peak 5 0.2 M

3

4

5

-

1

2

Peak 6 0.3 M

3

4

1

2

Peak 7 0.4 M

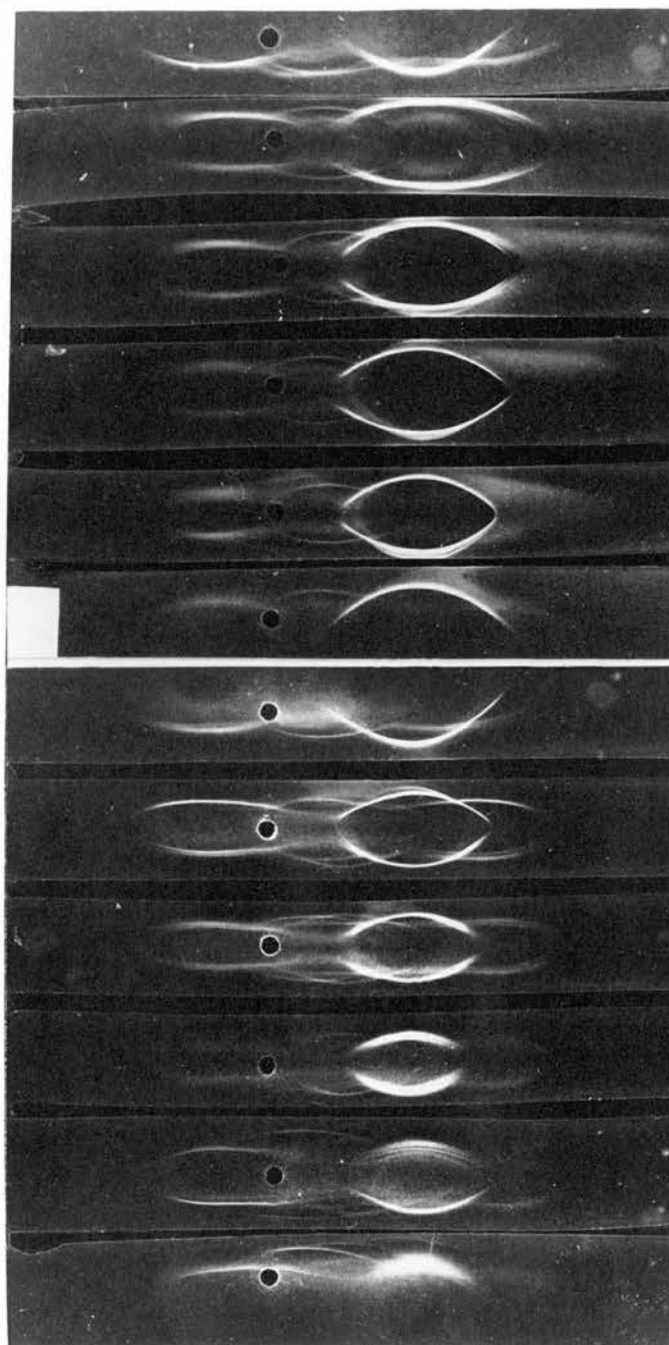
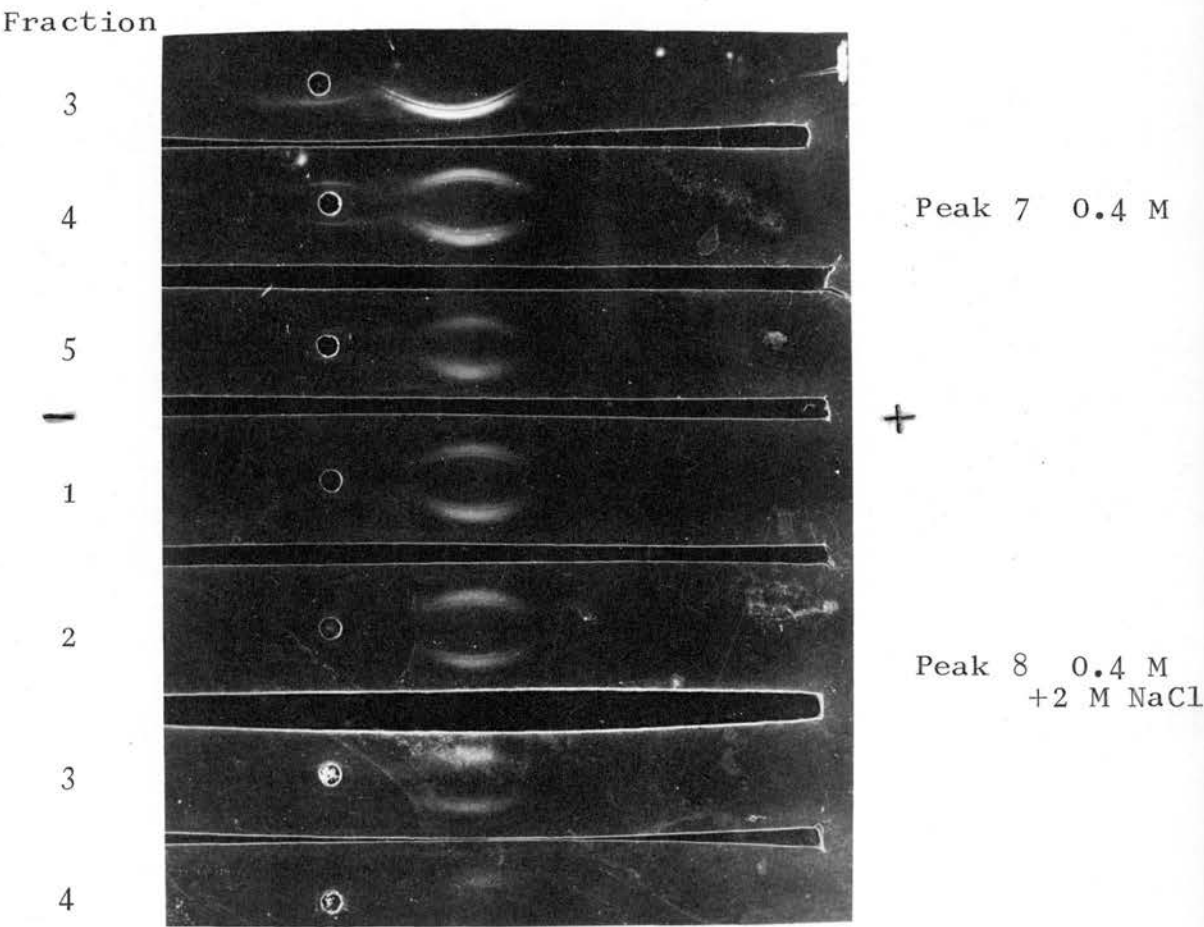


Figure 13. Immuno-electrophoretic analysis of chicken serum fractions eluted from DEAE cellulose.



emphasises the limitations of immunoelectrophoresis as a means of identifying unknown protein unless known specific antisera are used.

3. Attempts to demonstrate antibody activity in the 7S γ 1 protein.

a. Radioimmuno-electrophoresis studies: Adult White Leghorn hens were injected intravenously with 40 mg. BSA and bled 4 days, 6 days, 10 days and 14 days later. Radioimmuno-electrophoresis of these sera using anti-whole chicken serum and anti-7S γ 1 in the wells, and I^{125} BSA as antigen, showed antibody activity in the IgM arcs in the day 6 and day 10 sera, and in the IgG arc in day 10 and day 14 sera, but no activity was found in the 7S γ 1 arc in any of the samples (Fig. 15). Three more anti-BSA sera were then tested, two which had been taken 7 days after the second of two intravenous injections of 40 mg. BSA, and one six days after a single intravenous injection of 40 mg. BSA. Again, none showed antibody activity in the 7S γ 1 arc, whereas all three had IgG antibodies (Fig. 16). Although this method failed to demonstrate antibody activity in the 7S γ 1 protein, the results cannot be taken as proof that this is not an immunoglobulin. It is possible that the concentrations of labelled antigen or antibody were inadequate, or that these particular antisera did not have antibody activity in the

Figure 15.

The use of radioimmuno-electrophoresis to detect anti-BSA antibody activity in different chicken immunoglobulin classes.

- A. Straightforward immunoelectrophoresis slide.
- B. Developed film of the same slide after the addition of radiolabelled I^{125} BSA.

The troughs contain:

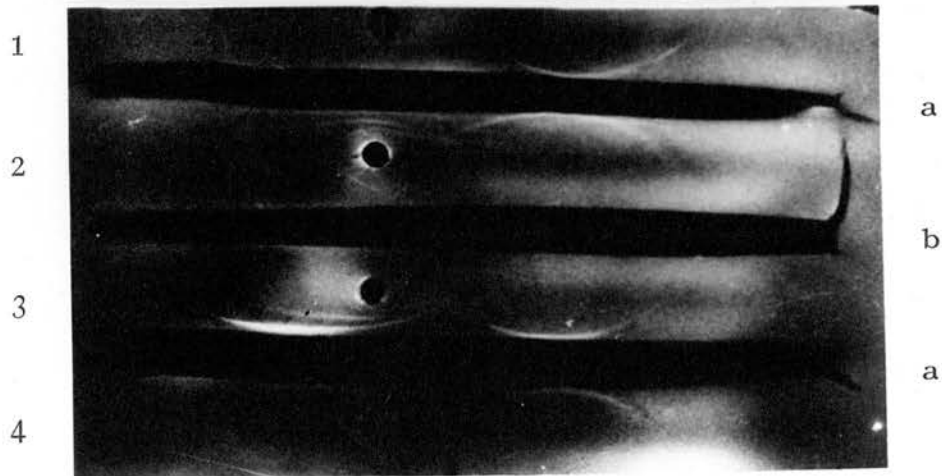
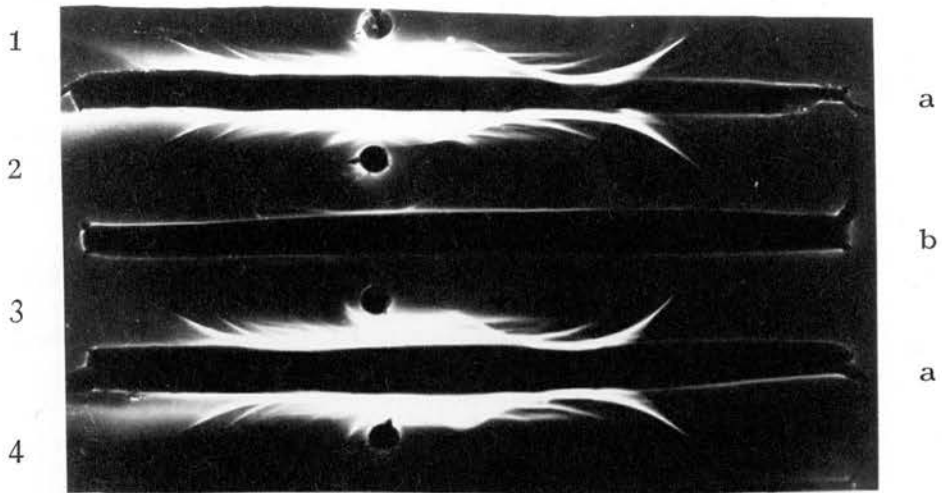
- a. anti-whole chicken serum;
- b. anti-7S γ 1 antiserum.

The wells contain:

- 1. chicken anti-BSA antiserum collected 4 days after inoculation;
- 2. chicken anti-BSA antiserum collected 6 days after inoculation;
- 3. chicken anti-BSA antiserum collected 10 days after inoculation;
- 4. chicken anti-BSA antiserum collected 14 days after inoculation.

Note - No antibody activity has been detected in 7S γ 1 arcs.

A



B

Figure 15

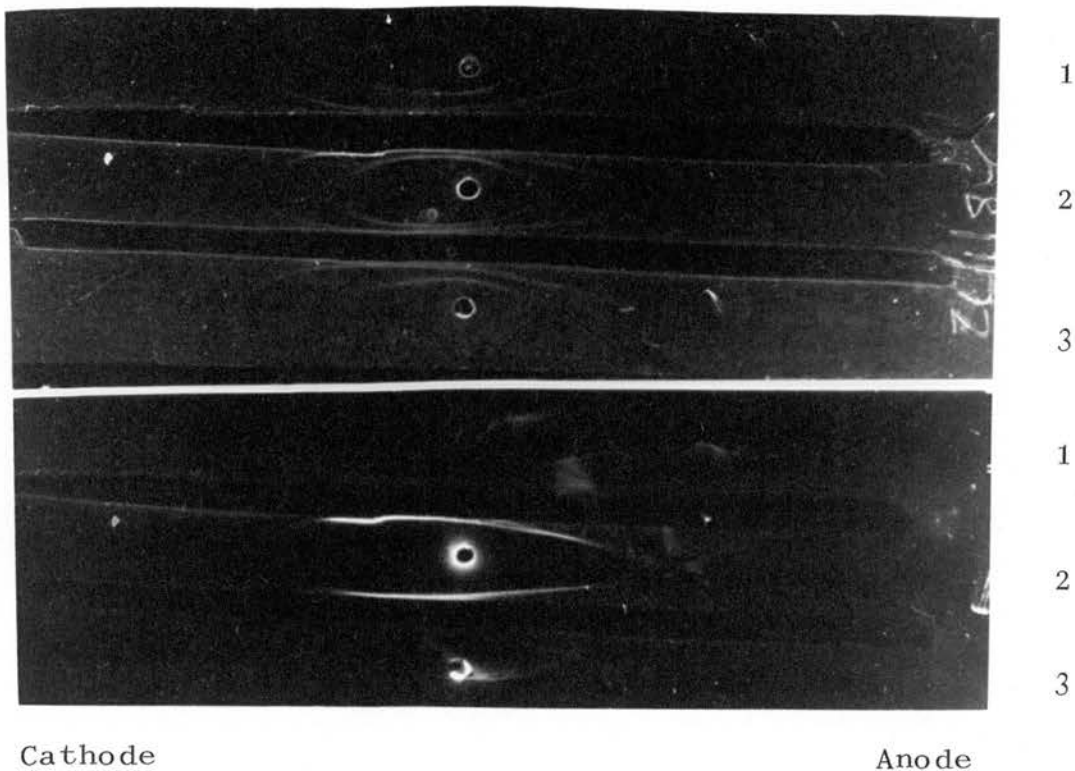


Figure 16.

The use of radioimmuno-electrophoresis to detect anti-BSA antibody activity in different chicken immunoglobulin classes.

All troughs contain polyvalent antiserum against IgG and 7S 1.

Well 1. Chicken anti-BSA antiserum 7 days after 2nd I/V injection of BSA.

Well 2. Chicken anti-BSA antiserum 7 days after 2nd I/V injection of BSA.

Well 3. Chicken anti-BSA antiserum 6 days after 1 injection of BSA.

Note - No antibody activity has been detected in the 7S γ 1 arcs.

7S γ 1 fraction. Dreesman et al., (1965) also using radioimmuno-electrophoresis demonstrated anti-protein activity in a γ 1A-globulin arc only in a few antisera, and suggested that the nature of the antigen influenced the type of antibody produced.

b. The use of anti-7S γ 1 antiserum as an antiglobulin reagent: For this purpose an indirect haemagglutination system using red cells sensitised with S.gallinarum polysaccharide and hyperimmune chicken anti-S.gallinarum antiserum was used. Bovine red cells were chosen for the test since it has been shown that some bovine cells do not agglutinate in a direct haemagglutination test (Gleeson-White et al., 1950; Spooner et al., 1970). Thus if a low titre of 7S γ 1 antibody was present in the serum, it would not be masked by direct agglutination of the red cells by IgG and IgM antibodies which would be expected to be present at high titres. Preliminary tests were first carried out to find the optimum concentration of polysaccharide for red cell sensitisation, using fowl erythrocytes. The optimum was found to be 0.2 ml. polysaccharide per 10 ml. of a 1.5% suspension of red cells and this concentration was used throughout subsequent tests. In this system the haemagglutination titre of the anti-S.gallinarum serum was 1/8192. Using bovine red cells the haemagglutination titre was 1/2. Control tubes contained (1) unsensitised cells + saline; (2) unsensitised cells + antiserum; (3) sensitised

cells + saline. All three were negative.

For the antiglobulin test, triplicate sets of dilutions of anti-S.gallinarum serum from 1/2 - 1/131,072 were set up and sensitised bovine red cells added to each. After incubation at 37°C until the red cells settled, the haemagglutination titre was read. This was 1/2 in each case. The cells were then washed three times in saline and to each tube of set (1) 1 drop of 1/40 rabbit anti-chicken IgM added, to set (2) 1 drop 1/20 rabbit anti-chicken IgG, and to set (3) 1 drop 1/20 rabbit anti-chicken 7S γ 1. The cells were again allowed to settle at 37°C and the antiglobulin titres read. No further agglutination had occurred in any of the sets. The cells were, therefore, washed another three times with saline, and 1 drop of 1/20 sheep anti-rabbit IgG added to each tube. After incubation the titres were read. The results are recorded in Table 1. It will be observed that agglutination occurred in all three sets - the titre in set (1) was 1/2048, in set (2) 1/512, and in set (3) 1/128. No agglutination occurred in control tubes containing sensitised cells + sheep anti-rabbit IgG, or sensitised cells + anti-S.gallinarum + sheep anti-rabbit IgG.

These results indicate that the 7S γ 1 protein may have antibody activity, and it is possible that S.gallinarum is a better antigen than BSA to stimulate production of this type of antibody. However, other

Table 1: The use of Anti-7S γ 1 antiserum as an antiglobulin reagent.

| Chicken anti-S.gallinarum reagent | HA titre | Antiglobulin titre with undernoted antiglobulin reagents | | | | |
|---|----------------------|--|----------------------|---|----------------------------|--|
| | | A-IgM $\frac{1}{40}$ | A-IgG $\frac{1}{20}$ | A-7S γ 1 $\frac{1}{20}$ unabsorbed | A- γ $\frac{1}{20}$ | A-7S γ 1 $\frac{1}{20}$ absorbed with IgG |
| Whole serum $\frac{1}{2}$ starting dilution | * -ve | $\frac{1}{2048}$ | $\frac{1}{512}$ | $\frac{1}{128}$ | NT | NT |
| DEAE cellulose 1 starting dilution | + -ve | NT | NT | NT | -ve | -ve |
| DEAE cellulose 1 starting dilution | + -ve | NT | NT | $\frac{1}{120}$ | $\frac{1}{160}$ | $\frac{1}{10}$ + |
| DEAE cellulose 1 starting dilution | + -ve | NT | NT | -ve | $\frac{1}{20}$ | -ve |
| DEAE cellulose $\frac{1}{30}$ starting dilution | + $\frac{1}{960}$ | NT | NT | $\frac{1}{7680}$ | NT | NT |

* = bovine red cells
+ = fowl red cells
+ = after addition of sheep anti-rabbit IgG
NT = not tested

interpretations of the results are also possible. With only one tube difference between titres with anti-7S γ 1 and anti-IgG, it could be that the anti-7S γ 1 had some anti-IgG activity, which, although too weak to be demonstrated by immunoelectrophoresis, would be picked up in the more sensitive antiglobulin test. Alternatively the antiserum might not contain any 7S γ 1 antibody to S.gallinarum, but the protein might still be an immunoglobulin with light chain determinants identical with those of IgG and IgM. The anti-7S γ 1 would then react with the light chains of IgG antibody molecules to cause agglutination.

In an attempt to rule out these latter possibilities, further antiglobulin tests were carried out, with the anti-7S γ 1 absorbed before use with IgG (0.01 M Phosphate elution peak from DEAE cellulose). S.gallinarum polysaccharide sensitised fowl red cells were used for these tests, and instead of whole anti-S.gallinarum antiserum, DEAE cellulose fractions which had been previously tested by SRD analysis for IgG and 7S γ 1 content. The fractions used were the 0.01 M peak (exclusively IgG), 0.09 M (IgG + 7S γ 1), 0.1 M (mainly IgG), and 0.2 M (IgG and IgM with trace 7S γ 1). The results are shown in Table 1. No antiglobulin haemagglutination occurred with either anti- γ (prepared by absorption of anti-IgG with serum from a bursectomised chicken containing IgM but not IgG, as

will be described in Section V), or anti-7S γ 1 with the 0.01 M (IgG) fraction. With the 0.09 M (IgG + 7S γ 1) fraction, the antiglobulin titre with anti- γ was 1/160, with unabsorbed anti-7S γ 1, 1/120, and with absorbed anti-7S γ 1 slight agglutination occurred at 1/10. No agglutination occurred with anti-7S γ 1 with the 0.1 M fraction, whilst with the 0.2 M fraction containing mainly IgM the antiglobulin titre with unabsorbed anti-7S γ 1 was 1/7680.

The implication from these results is that the anti-7S γ 1 antiglobulin activity observed in the original haemagglutination experiments was due either to anti-light chain or anti-IgG activity. Therefore, at the present time, the evidence is against 7S γ 1 being an immunoglobulin.

DISCUSSION

These experiments have proved valuable in establishing a method of preparation of the immunoglobulins which are to be studied from various aspects in the following sections of this thesis.

A third γ component has also been isolated, and, although it has not been possible to demonstrate that it is immunoglobulin in nature, some of its physico-chemical properties have been defined. This should prove of value in subsequent investigations into other chicken immunoglobulin classes.

Other attempts were also made to isolate additional immunoglobulins from chicken secretions, but since they were unsuccessful, they have not been recorded in detail in this thesis. However, brief mention can be made of them here.

The first attempt was to prepare a γ -globulin from chicken intestinal secretions by a method used in the preparation of mammalian IgA - i.e. DEAE cellulose fractionation of gut contents. A buffer system of increasing NaCl from 0.01 M to 0.3 M, in 0.01 M phosphate pH 7.6 was used. A fairly high 0.125 M peak was obtained and this was tested by immunoelectrophoresis with anti-whole chicken serum and anti-chicken IgG. A very faint line in the β_1 region appeared, and so the material was injected into a rabbit to produce an antiserum. The antiserum which was obtained, however, had no activity against sodium sulphate precipitated intestinal globulins or bile, but gave a line in the α region when tested by immunoelectrophoresis against unpurified gut contents.

Chicken tears were used in a further attempt to demonstrate a third antibody class in chicken secretions. Small quantities of tears were collected from normal birds and from birds which had had BSA in Freund's complete adjuvant administered directly into the eyes. Two administrations were given, with 1 month's interval, and tears and serum collected 1, 4 and 7 days after the

second antigen dose.

Immuno-electrophoretic analysis of the tears revealed the presence of IgG, IgM and other γ components (Fig. 17, 18) but when radioimmuno-electrophoresis was carried out with ^{125}I labelled BSA, no antibody could be demonstrated in any of the samples of tears, although some IgG antibody was present in the sera. Since tears can only be collected in minute quantities other methods for the isolation of γ components, such as sodium sulphate precipitation and chromatography, etc. were not possible.

Since the work for this thesis was completed a report has appeared in the literature (Lebacqz-Verheyden et al., 1972) where a third immunoglobulin, tentatively named IgA, has been isolated from chicken intestinal secretions and bile. Its immuno-electrophoretic pattern was similar to that described for a third anti-ferritin antibody by Patterson et al., (1965), and an anti-human serum albumin antibody by Dreesman et al., (1965). It migrated faster than the 7S γ 1 described here, and had a bimodal distribution. A component resembling it can be faintly seen in chicken tears. Physico-chemical characterisation studies are to be reported by these authors.

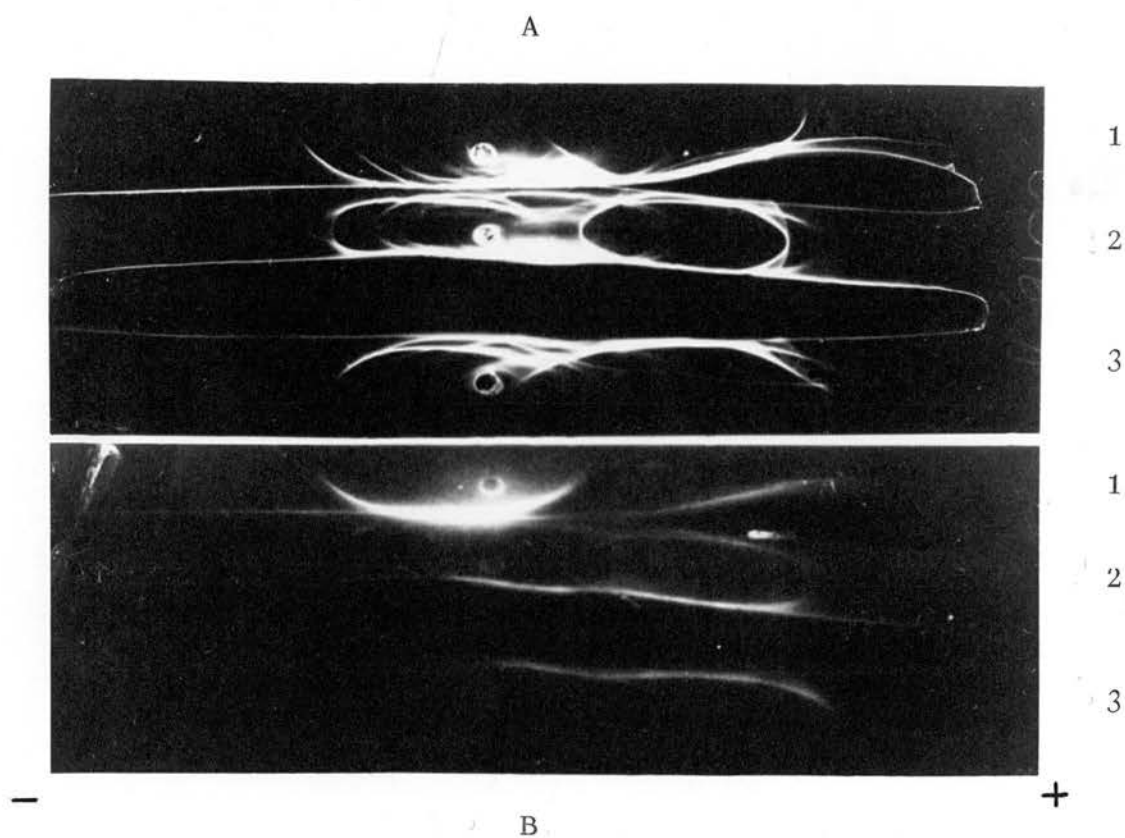


Figure 17.

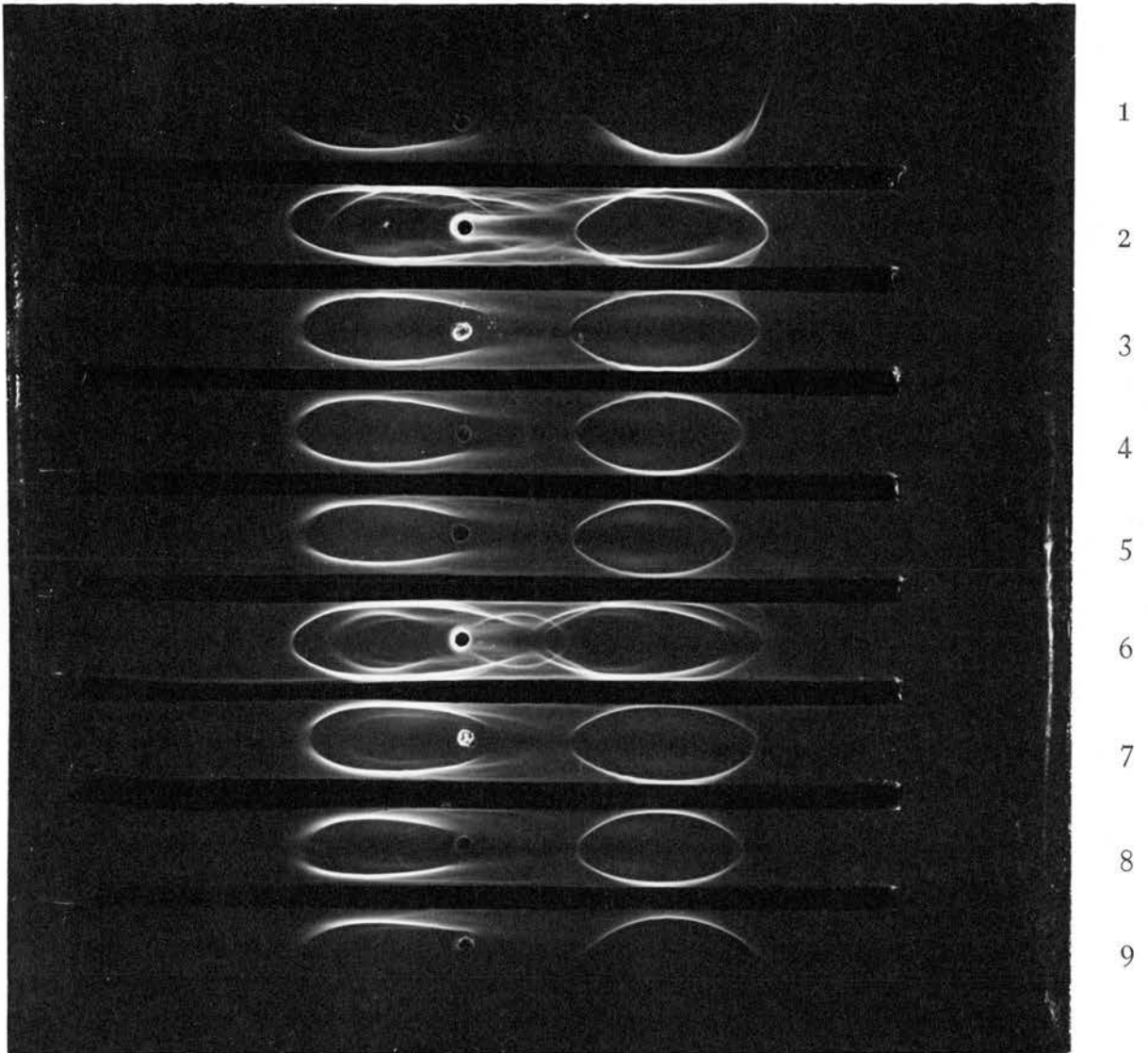
The use of radioimmuno-electrophoresis in an attempt to detect the presence of antibodies in chicken tears.

- A. Straightforward immunoelectrophoresis slide.
- B. Developed film of the same slide after the addition of radiolabelled I^{125} BSA.
- 1. Chicken anti-BSA serum 7 days after administration of antigen into the eyes.
- 2. Chicken tears collected 4 days after antigen.
- 3. Chicken tears collected 7 days after antigen.

SECTION III

**STUDIES ON THE ONTOGENY OF
IMMUNOGLOBULIN SYNTHESIS:
THE EFFECTS OF BURSECTOMY.**

Figure 18



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SECTION III

**STUDIES ON THE ONTOGENY OF
IMMUNOGLOBULIN SYNTHESIS:
THE EFFECTS OF BURSECTOMY.**

Since the discovery by Glick et al. in 1956 that antibody responses in the fowl were in some way dependent on the bursa of Fabricius, bursectomy has become a widely used experimental technique.

Subsequent to the characterisation of chicken immunoglobulins carried out in the previous section, the procedure of bursectomy was adopted to investigate various aspects of the synthesis of the immunoglobulin classes in chickens.

PART I

THE BURSECTOMY TECHNIQUE

INTRODUCTION

Several different methods of bursectomy have been described in the literature. In the original experiments the bursa was removed surgically at 2 weeks of age (Glick et al., 1956; Chang et al., 1957, 1959). This resulted in a marked inhibition of antibody responses to sheep erythrocytes and S.typhimurium. When the operation was carried out on 5 or 10 week old birds on the other hand, there was little or no effect on antibody responses to foreign red cells (Chang et al., 1957) or bovine serum albumin (Mueller et al., 1962). Experiments by Graetzer et al., (1963), Chang et al., (1957) and Mueller et al., (1960, 1962) confirmed this relationship between the time of bursectomy and the degree of inhibition of antibody production, and most workers subsequently removed the

bursa on the first day of life (Cooper et al., 1966a; Arnason and Jankovic, 1967; Jankovic and Isakovic, 1967; Dent et al., 1968).

Even neonatal bursectomy does not completely eliminate humoral responsiveness, however, for such birds can mount secondary responses after repeated antigenic stimulation (Claflin et al., 1966; Jankovic and Isakovic, 1966, 1967; Arnason and Jankovic, 1967) and have been shown to produce natural haemagglutinins to rabbit red cells by the age of 5 months (Jankovic and Isakovic, 1967). Nor are the serum immunoglobulins eliminated by neonatal surgical bursectomy. In fact they are often present in these birds in normal amounts. However, Arnason and Jankovic, (1967) have reported a late developing IgG deficiency while IgM levels remained normal, and Cooper et al., (1966a) found a lowering of IgG levels coupled with higher than normal IgM levels in some of their birds 7 weeks after neonatal bursectomy. Conversely, Van Meter et al., (1969) reported that neonatally bursectomised chicks after a slight delay produced higher than normal levels of IgG but normal amounts of IgM. Thus, as far as serum immunoglobulin levels are concerned, this method of bursectomy produces very inconsistent results.

Because of this Cooper et al., (1965, 1966a) combined neonatal surgical bursectomy with sub-lethal whole body X-irradiation. This proved to be a much more efficient method for producing both antibody and immunoglobulin

with higher than normal IgM levels. Approximately 50% had severely depressed IgG levels which were less than 1% of the normal.

Another method of bursectomy which has been reported is X-irradiation of the bursa itself (Weber and Weidanz, 1969). Here the bursa was given a dose of 1000 R whilst the rest of the body was shielded, once at 1 day old and again at 1 week old. The effects on antibody production were similar to neonatal surgical bursectomy - that is depressed primary responses but almost normal secondary responses, and serum immunoglobulins were normal in most cases. This method has the disadvantage of causing faecal matting around the vent, and a stunting effect on body growth.

These side-effects are also observed after hormonal bursectomy, especially when large doses of testosterone are given early in embryonic development. Both this method, and the bursectomy/irradiation method also have the added disadvantage of a lack of specificity. The injection of testosterone even as late as the 12th day of incubation causes atrophy of the thymic cortex in 40% of birds so treated. The degree of degeneration varies between individuals, but 10% show a complete cortical atrophy with degeneration also occurring in the medulla by the age of 4 weeks (Warner et al., 1962). Irradiation may also have a far reaching although less permanent effect on the other lymphoid organs of the body.

Thus none of these methods of bursectomy achieve the ideal requirement of complete inhibition of bursal function without damage to other organs. A closer approach to these ideals has been reported to be attained by the method of surgical bursectomy in ovo, provided that this is performed early enough in embryonic development (Cooper et al., 1969). It was therefore decided to use this particular technique in the present work.

At this time the method has been described only very briefly by Van Alten et al., (1968), and has not been used in other laboratories, probably because of the technical problems involved. The first requirement, therefore, was to develop this technique to a degree of proficiency which would allow a reasonable survival rate.

PROCEDURE

The evidence of Cooper et al., (1969) suggested that the operation should be carried out on the 17th day of incubation or earlier for marked inhibition of both IgG and IgM synthesis, but for the initial attempts it was decided to use 18 day old embryos.

A pilot experiment was first carried out in which 30 18 day old embryos were bursectomised surgically without prior candling or marking, the position of the shell opening being chosen at random. Each egg was numbered, the position and degree of manipulation of the embryo

and yolk sac carefully documented, and a note kept of which chickens hatched. It was found that only those chickens which had by chance been in such a position that their tail regions were more or less directly under the shell opening, and thus suffered minimum interference, hatched. If the embryo had to be manoevered or the yolk sac handled the embryo rarely survived. It was necessary therefore to be able to recognise anatomical features of the embryo through the shell by candling so that the correct dorsal approach was always made. This was achieved by a process of trial and error. Several hundred eggs were candled and carefully studied for common recognisable features. An oval dense area with a smooth and regular edge could be picked out and was at first thought to be the embryo's back. Several shells were marked above this point and a 1 x 2 cm. opening made. In each case the yolk sac was found uppermost - i.e. the opening had been made on the opposite side of the egg to that required. A second series of eggs were then marked as before, and the openings made directly opposite the mark. In 95% of these the back of the embryo was found below the hole.

The final operative technique which was used for all subsequent experiments is illustrated in Figs. and was carried out as follows:

Equipment (Fig. 19)

1. Egg candler.
2. Dental drill with carborundum disc.
3. Clamp and plasticine.

4. 1 Pair Silcock's curved iris forceps.
5. 1 Pair $4\frac{1}{2}$ ins. suture and dissecting forceps.
6. 1 Pair Graefe's fixation forceps.
7. 1 Pair intracapsular Arruga's forceps.
8. 1 Pair spring action, curved Westcott's scissors.
9. 1 ins. broad sellotape.
10. Swabs.
11. Surgical spirit.

(Items 4 to 8 supplied by C. W. Dixey & Sons Ltd., 19 Wigmore Street, London, W.1.)

Outbred White Leghorn fertile hatching eggs were obtained from the East of Scotland College of Agriculture and were incubated up to the time of operation in a rotatory egg incubator (Westernette).

Method

The egg was candled to ensure that the embryo was alive and to mark the position of the tail region. Individual features of the embryo are not easily distinguished at the 17th or 18th day of incubation but as already mentioned an oval dense area with a regular edge can be recognised (Fig. 20a). Here the edge of the air space appears convex. When this position had been found the egg was rotated on its short axis through 180° and a $1\frac{1}{2}$ cm. x 1 cm. rectangle marked on it as shown in Fig. 20b. The edge of the air space appears concave in this position and large blood vessels can be distinguished. The shell was then swabbed with

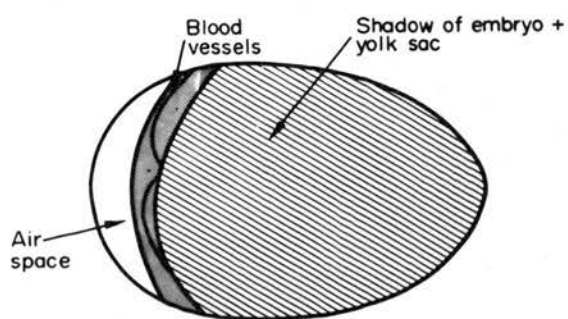
Figure 19.

Instruments and equipment required for in ovo bursectomy.

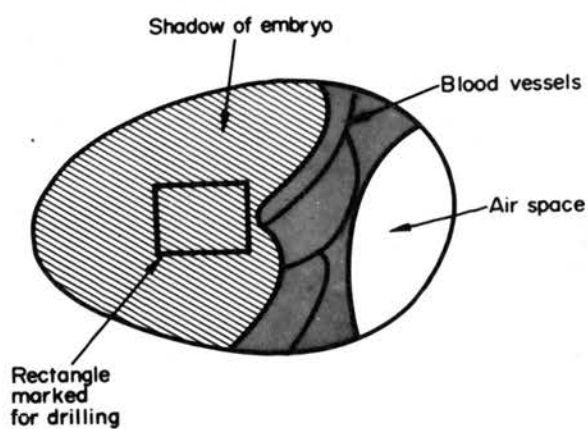
Figure 20.

Appearance of the embryonated egg during candling prior to in ovo bursectomy.

- a) First position located, showing convex edge to air-space and large shadow of embryo.
- b) Egg after rotation through 180° on short axis from the first position. Note the concave edge to the air-space. The site to be marked for cutting the shell is indicated.



(a)



(b)

surgical spirit, and with the egg held so that the pencil mark was uppermost at all times, the rectangle of shell was cut out with a dental drill, without penetrating the shell membrane.

The egg was placed in the clamp with the pointed end towards the operator, the surface swabbed again with spirit, and the rectangle of shell removed (Fig. 21). The shell membrane was then gently torn back taking care not to rupture the underlying chorioallantoic membrane which would cause haemorrhage (Fig. 22). With the suture and dissecting forceps a small opening was made in this membrane avoiding large blood vessels, and the embryo was grasped gently and eased round until its tail could be grasped firmly with the fixation forceps and pulled through the opening. The tail region was generally located towards the bottom left hand corner of the rectangular area. The handle of the forceps was anchored in the plasticine attached to the clamp in such a position that the embryo's cloacal region was clear of the fluid and easily accessible for surgery (Fig. 23). The dorsal lip of the cloaca was then grasped with the Arruga's forceps and using the small spring action scissors, a 3 mm. long horizontal incision was made between the vent and the base of the tail (Fig. 24). The bursa of Fabricius could now be seen lying subcutaneously. The dorsal surface of the bursa was freed by blunt dissection and the point of the

Figure 21.

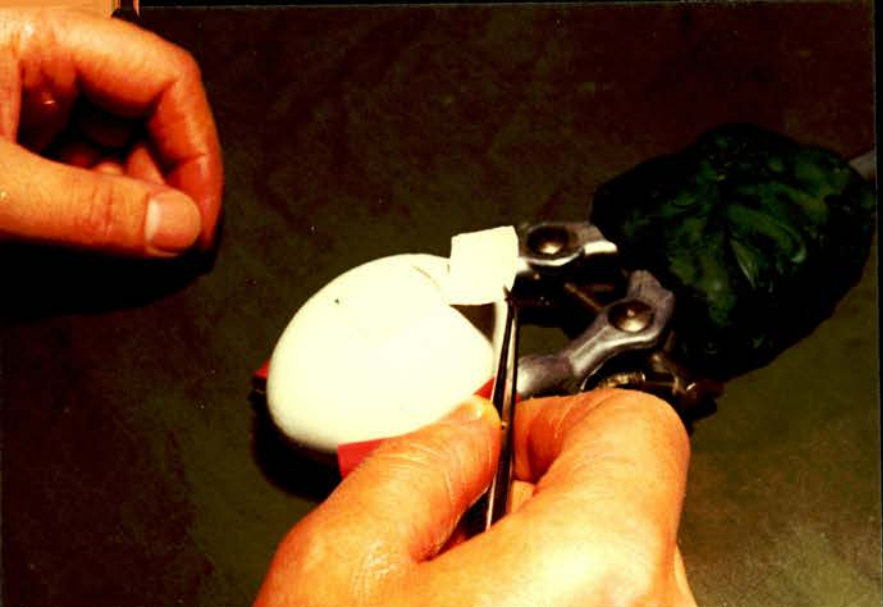
Lifting off the rectangle of shell.

Figure 22.

Removal of shell membrane.

Figure 23.

The embryo in a suitable position for surgery.
Note the tail grasped with Graefe's forceps
which are anchored in plasticine.



scissors was slipped gently over and in front of the bursa to draw it back through the incision (Fig. 25). The bursa was then gently grasped near its stalk with the Arruga's forceps, and, taking care not to rupture the capsule, was removed intact by gentle traction. Traction was used rather than dissection in order to minimise the risk of cutting the ureters which run along each side of the bursa and open into the cloaca close to the bursal duct, but occasionally it was necessary to cut through the duct connecting the bursa to the rectum (Fig. 26). The tail was then released and the embryo returned to its original position in the egg. No suturing was necessary as the incision healed without scar by the time the chick hatched. The opening in the shell was sealed with sellotape (Fig. 27) and the egg returned to a stationary incubator where it was wedged firmly in position with the opening uppermost to avoid any leakage of fluid.

The operation was carried out as aseptically as possible under a sterile hood (Luckham's inoculation cabinet).

The earliest attempts gave a success rate (birds hatched/birds operated on) of only about 10% but after some practice this rose to 70%. With practice the whole procedure could be completed in 5 minutes, and the efficiency of complete removal of the bursa, i.e. without damaging the bursa or rupturing its capsule, approached 100%.

Figure 24.

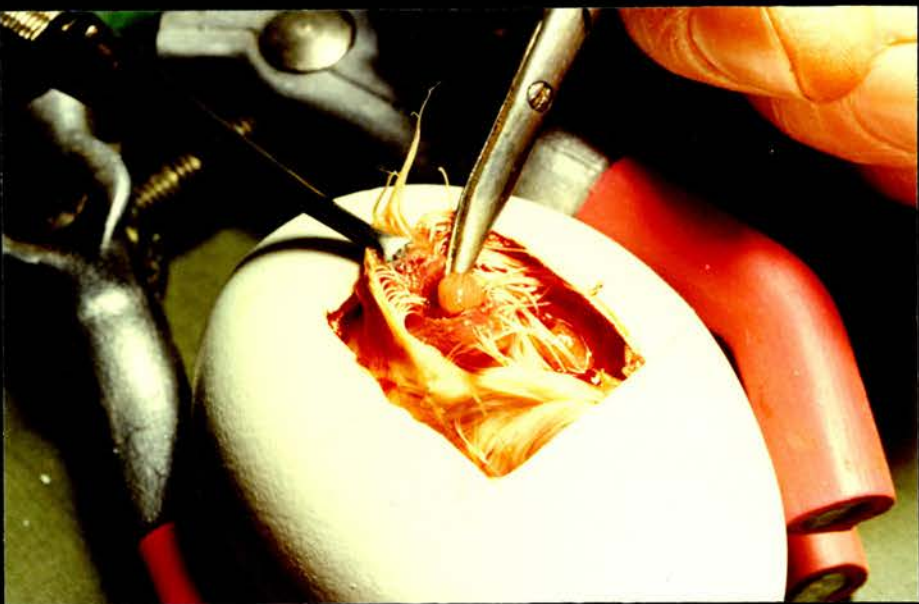
Horizontal incision being made between the tail and the vent.

Figure 25.

Bursa drawn forward through the incision.

Figure 26.

Removal of the bursa by gentle traction and blunt dissection.



Chicks hatched unaided on the 21st day of incubation (Fig. 28), but occasionally the sellotape had to be loosened if the chick began "pipping" underneath it.

Figure 27.

**The opening in the shell sealed with sellotape.
Bursa shown undamaged after removal.**

Figure 28.

Bursectomised chicks hatching.



PART II

ONTOGENY OF IMMUNOGLOBULIN SYNTHESIS IN NORMAL BIRDS AND
BIRDS BURSECTOMISED SURGICALLY IN OVO

INTRODUCTION

Once the technique of surgical bursectomy in ovo had been developed and good hatching rates obtained, the next requirement was to evaluate its effects on immunoglobulin synthesis.

In their original experiments with embryonic bursectomy carried out on the 19th day of incubation, Van Alten et al., (1968) found that IgG synthesis was severely depressed in all these birds, but IgM levels remained normal. The immunoglobulin levels were assessed at 5 and 6 weeks of age by immunoelectrophoretic analysis only. In later experiments the same group of workers bursectomised groups of birds as 17, 18, 19 or 20 day old embryos (Cooper et al., 1969). All the groups had depressed IgG levels at 6 weeks of age and some chickens from each group were also deficient in IgM. However, the mean IgM levels were only depressed in the 17 day group. A few birds were totally agammaglobulinaemic, and the authors suggested that the critical time for bursectomy to abolish IgM synthesis was around the 17th day of incubation.

On closer inspection their findings, which were based on limited numbers of birds, did not appear to be statistically significant. In order to see if these

results could be repeated a more comprehensive study of immunoglobulin synthesis in embryonically bursectomised birds was undertaken.

MATERIALS AND METHODS

Bursectomy: Bursectomy was carried out surgically, as already described, on the 17th, 18th or 19th day of incubation. Bursectomised chicks and controls hatched from the same batches of eggs were reared together under conventional conditions.

Blood sampling: Chicks were blood sampled at 1 day old by heart puncture, and thereafter at weekly intervals by wing vein stab. Only 0.4 ml. was collected from day old chicks, and 1-2 ml. from older birds. The blood was allowed to clot, and the serum separated and stored at -20°C until testing.

Serum immunoglobulin measurements: Serum IgG, IgM and γS levels were measured by the single radial immunodiffusion (SRD) method already described.

Post Mortem examination: Chickens which died during the experimental period were examined for bursal remnants, and sections of spleen, caecal tonsil and thymus were fixed in Carnoy's fluid for 30 minutes and then preserved in 90% alcohol for future histological examination.

Birds which survived to 19 weeks of age were killed by intravenous injection of pentobarbitone and examined for bursal remnants. The spleen, caecal tonsil, thymus and Harderian gland were kept for histology.

Those birds which were found to have bursal remnants were excluded from the immunoglobulin studies.

RESULTS

Serum IgG and IgM levels from hatching until 19 weeks of age in normal chickens, and chickens which were bursectomised on the 17th, 18th or 19th day of embryonic development are shown in tables 2, 3 and 4, 5 respectively.

In normal birds IgM synthesis began immediately after hatching and serum IgM levels rose steadily to reach a peak at about 40 days of age, then dipped slightly before reaching a plateau at 60 days at the normal adult level. Serum IgG levels were approximately 60% of the adult level at hatching but began to fall immediately until at 20 days the level was only approximately 10% of the adult pool. This fall was due to catabolism of maternal IgG before active synthesis began. The IgG level then rose steadily, although more slowly than IgM, to reach a peak about 90% of the reference pool at around 65 days. It then fluctuated between 60% and 100% of the reference value until the end of the test period. The dip in the IgM level occurred as the IgG was reaching its peak.

This pattern was very similar to that reported for normal chickens by Van Meter et al., (1969). They also noted a dip in IgM production which corresponded with

Table 2 : Serum Immunoglobulin levels in normal chickens.

| Chicken No. | 112 | | 128 | | 181 | | 184 | | 186 | | 249 | | 245 | |
|-------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Age | IgG % | IgM % | IgG % | IgM % | IgG % | IgM % | IgG % | IgM % | IgG % | IgM % | IgG % | IgM % | IgG % | IgM % |
| 1 day | 24 | -ve | 34 | -ve | 62 | -ve | 34 | -ve | 68 | -ve | 57 | -ve | 60 | -ve |
| 1 wk | 25 | 9 | 24 | 13 | 48 | 12 | 34 | 11 | 39 | 33 | 36 | 11 | 26 | |
| 2 wks | 11 | 25 | 34 | 17 | 17 | 24 | 13 | 43 | 6 | 40 | 20 | 58 | 16 | 56 |
| 3 wks | 12 | 40 | 12 | 40 | 12 | 49 | 14 | 69 | 7 | 71 | 17 | 77 | 13 | 70 |
| 4 wks | 13 | 47 | 8 | 36 | 13 | 50 | 9 | 50 | 8 | 55 | 21 | 97 | 21 | 94 |
| 5 wks | 14 | 51 | 8 | 35 | 17 | 104 | 13 | 95 | 8 | 98 | 12 | 98 | 16 | 102 |
| 6 wks | 14 | 62 | 11 | 50 | 36 | 125 | 10 | 103 | 11 | 107 | 15 | 105 | 14 | 72 |
| 7 wks | 26 | 74 | 15 | 90 | 48 | 113 | 28 | 109 | 62 | 112 | 43 | 110 | 55 | 90 |
| 8 wks | 17 | 41 | 23 | 76 | 66 | 101 | | | 45 | 109 | 43 | 94 | 33 | 102 |
| 9 wks | 19 | 30 | 26 | 40 | 94 | 110 | 50 | 126 | | | 86 | 116 | | |
| 10 wks | 33 | 46 | 70 | 80 | 83 | 106 | 51 | 121 | 60 | 110 | 104 | 149 | 36 | 95 |
| 11 wks | 49 | 17 | 40 | | 72 | 109 | | | | | | | 30 | 70 |
| 12 wks | 29 | 20 | | 27 | | | 54 | 101 | 64 | 106 | | | | |
| 13 wks | 14 | 11 | 50 | 72 | 98 | 104 | 55 | 102 | 62 | 105 | 94 | 121 | | |
| 14 wks | | | 52 | 73 | 100 | 103 | 58 | 101 | 60 | 107 | 68 | 133 | | |
| 15 wks | | | 49 | 74 | | | 79 | 106 | 75 | 102 | | | 65 | 100 |
| 16 wks | | | | | 111 | 100 | 106 | 110 | 73 | 96 | 50 | 110 | | |
| 17 wks | | | | | 94 | 78 | 76 | 101 | 71 | 74 | | | | |
| 18 wks | | | | | 87 | 73 | 79 | 103 | 83 | 96 | | | 76 | 130 |
| 19 wks | | | | | 85 | 69 | 85 | 104 | 106 | 107 | | | | |

Table 3: Serum Immunoglobulin levels in chickens bursectomised as 17 day old embryos.

| Chicken No. | 213 | | 222 | | 217 | | 220 | | 225 | |
|-------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| | IgG % | IgM % | IgG % | IgM % | IgG % | IgM % | IgG % | IgM % | IgG % | IgM % |
| 1 day | 43 | -ve | 62 | -ve | 68 | -ve | 55 | -ve | 39 | -ve |
| 1 wk | 19 | -ve | 36 | -ve | 28 | -ve | 31 | -ve | 22 | -ve |
| 2 wks | 8 | 9 | 11 | 9 | 8 | 8 | 7 | -ve | 11 | 10 |
| 3 wks | 4 | 28 | 2 | 25 | 2 | 5 | 4 | 12 | <1 | 15 |
| 4 wks | 25 | 60 | <1 | 63 | 3 | 6 | <1 | 103 | <1 | 56 |
| 5 wks | 12 | 52 | <1 | 102 | <1 | 4 | 2 | 114 | <1 | 39 |
| 6 wks | 9 | 18 | <1 | 114 | 1 | 4 | 5 | 61 | <1 | 48 |
| 8 wks | 46 | 105 | <1 | 107 | 2 | -ve | -ve | 130 | 2 | 42 |
| 11 wks | 106 | 130 | <1 | 39 | 2 | -ve | -ve | 121 | <1 | 30 |
| 15 wks | 124 | 137 | <1 | 13 | <1 | -ve | -ve | 148 | 2 | 20 |
| 18 wks | 121 | 156 | <1 | 6 | <1 | -ve | -ve | 200 | <1 | 6 |

Table 4 : Serum Immunoglobulin levels in chickens bursectomised as 18 day old embryos.

| Chicken No. | 115 | 117 | 144 | 145 | 147 | 148 | 116 | 118 | 121 |
|-------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Age | IgG % Igm % | IgG % Igm % | IgG % Igm % | IgG % Igm % | IgG % Igm % | IgG % Igm % | IgG % Igm % | IgG % Igm % | IgG % Igm % |
| 1 day | 65 -ve | 39 -ve | 57 -ve | 21 -ve | 33 -ve | 24 -ve | 57 -ve | 34 -ve | 17 -ve |
| 1 wk | | | 24 -ve | | 46 -ve | 27 | | | |
| 2 wks | 5 5 | 5 -ve | 4 6 | 8 8 | 10 5 | 4 7 | 3 6 | 8 5 | 5 20 |
| 3 wks | 2 19 | 2 5 | <1 38 | 3 22 | 2 7 | 2 28 | 1 37 | 4 53 | 5 65 |
| 4 wks | <1 12 | <1 8 | 2 100 | 3 73 | <1 14 | 6 80 | <1 94 | 3 65 | 9 65 |
| 5 wks | <1 12 | <1 7 | 9 109 | 1 30 | <1 19 | 14 84 | <1 65 | 9 107 | 9 74 |
| 6 wks | <1 12 | <1 5 | 4 87 | 2 35 | <1 14 | 14 102 | 1 91 | 17 101 | 12 101 |
| 7 wks | <1 9 | <1 -ve | 17 117 | 7 39 | <1 9 | 31 103 | 2 68 | | 10 |
| 8 wks | <1 10 | <1 <1 | | | | | 15 48 | 16 34 | 32 95 |
| 9 wks | <1 <1 | -ve <1 | 32 108 | 8 40 | <1 8 | 73 124 | 15 86 | | |
| 10 wks | <1 9 | <1 <1 | 66 123 | 14 62 | <1 8 | 76 118 | 13 92 | 34 70 | 34 168 |
| 11 wks | <1 9 | <1 <1 | 76 127 | 11 57 | <1 9 | 84 117 | 20 98 | 40 50 | 44 108 |
| 12 wks | <1 8 | <1 <1 | | 8 47 | <1 6 | 102 112 | 23 47 | 46 44 | 57 144 |
| 13 wks | <1 8 | <1 -ve | 106 108 | | <1 9 | 104 117 | 34 104 | 60 77 | 92 58 |
| 14 wks | | <1 <1 | | | | | | | 98 |
| 15 wks | <1 8 | <1 <1 | | | <1 6 | | 40 106 | 92 103 | 88 |
| 16 wks | <1 6 | <1 <1 | 102 117 | | <1 6 | 120 138 | 51 105 | 52 72 | 90 83 |
| 17 wks | | <1 -ve | 102 118 | | <1 5 | 122 126 | 103 | 90 134 | 96 116 |
| 18 wks | | | | | <1 6 | | | | |
| 19 wks | | | 33 100 | | <1 5 | 106 125 | | | |

Table 5 : Serum Immunoglobulin levels in chickens bursectomised as 19 day old embryos.

| Chicken No. | 171 | 170 | 176 | 175 | 163 | 164 | 165 | 166 |
|-------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Age | IgG % IgM % | IgG % IgM % | IgG % IgM % | IgG % IgM % | IgG % IgM % | IgG % IgM % | IgG % IgM % | IgG % IgM % |
| 1 day | 67 -ve | 33 -ve | 39 -ve | 37 -ve | 68 -ve | 81 -ve | 43 -ve | 56 -ve |
| 1 wk | 89 6 | 13 -ve | 20 7 | | 62 -ve | 32 -ve | 35 6 | 40 4 |
| 2 wks | 4 27 | <1 5 | <1 14 | 7 21 | 14 43 | 4 7 | 9 34 | 4 5 |
| 3 wks | | <1 12 | <1 21 | 3 22 | 1 80 | <1 60 | 11 43 | |
| 4 wks | 5 55 | <1 15 | 2 101 | 2 29 | | <1 98 | 13 60 | <1 5 |
| 5 wks | 4 98 | <1 20 | 2 87 | <1 25 | <1 102 | <1 101 | | <1 6 |
| 6 wks | 7 98 | | | <1 48 | -ve 105 | <1 121 | 29 116 | <1 8 |
| 7 wks | | <1 18 | <1 53 | <1 39 | -ve 111 | <1 121 | 29 124 | <1 5 |
| 8 wks | 32 101 | <1 39 | <1 100 | | -ve 104 | | 29 113 | <1 5 |
| 9 wks | 36 100 | | | 3 86 | | <1 146 | 26 68 | <1 5 |
| 10 wks | 39 107 | <1 12 | <1 65 | | | | | <1 4 |
| 11 wks | | | | 19 92 | | 1 140 | 68 115 | <1 3 |
| 12 wks | 44 103 | <1 9 | 2 92 | 16 123 | | 2 146 | 68 115 | -ve -ve |
| 13 wks | 47 119 | <1 7 | <1 32 | 6 108 | | 1 136 | | |
| 14 wks | | <1 7 | <1 21 | 22 120 | | | 68 109 | -ve -ve |
| 15 wks | 101 119 | <1 5 | <1 13 | 4 122 | | 1 133 | 29 31 | |
| 16 wks | | | | <1 120 | | | | -ve -ve |
| 17 wks | 121 128 | <1 4 | <1 9 | | | <1 124 | 23 7 | -ve -ve |
| 18 wks | 111 140 | <1 4 | <1 7 | | | | | -ve -ve |
| 19 wks | 78 128 | | | | | <1 114 | 14 5 | |

the peak in IgG.

On inspection of the results obtained for the bursectomised birds, it became apparent that immunoglobulin synthesis in these chickens fell into three distinct patterns. These are shown in Fig. 29 where immunoglobulin levels for three individual birds representing the three types of pattern are plotted in graphic form along with the normal.

The first group represented in Fig. 29b synthesised both IgG and IgM and their serum immunoglobulin development was very similar to the normal, although IgG rose rather more slowly to the adult level.

Other birds as represented in Fig. 29c after a delay of about 7 days developed IgM levels which exceeded the normal but failed to produce IgG, their serum IgG level falling to zero as maternal IgG was eliminated. The decreased catabolic rate for IgG in bursectomised hypogammaglobulinaemic chickens reported by Frommel et al., (1970) has not been seen in this experiment. Maternal IgG levels in bursectomised groups fell as quickly as in the normal chickens.

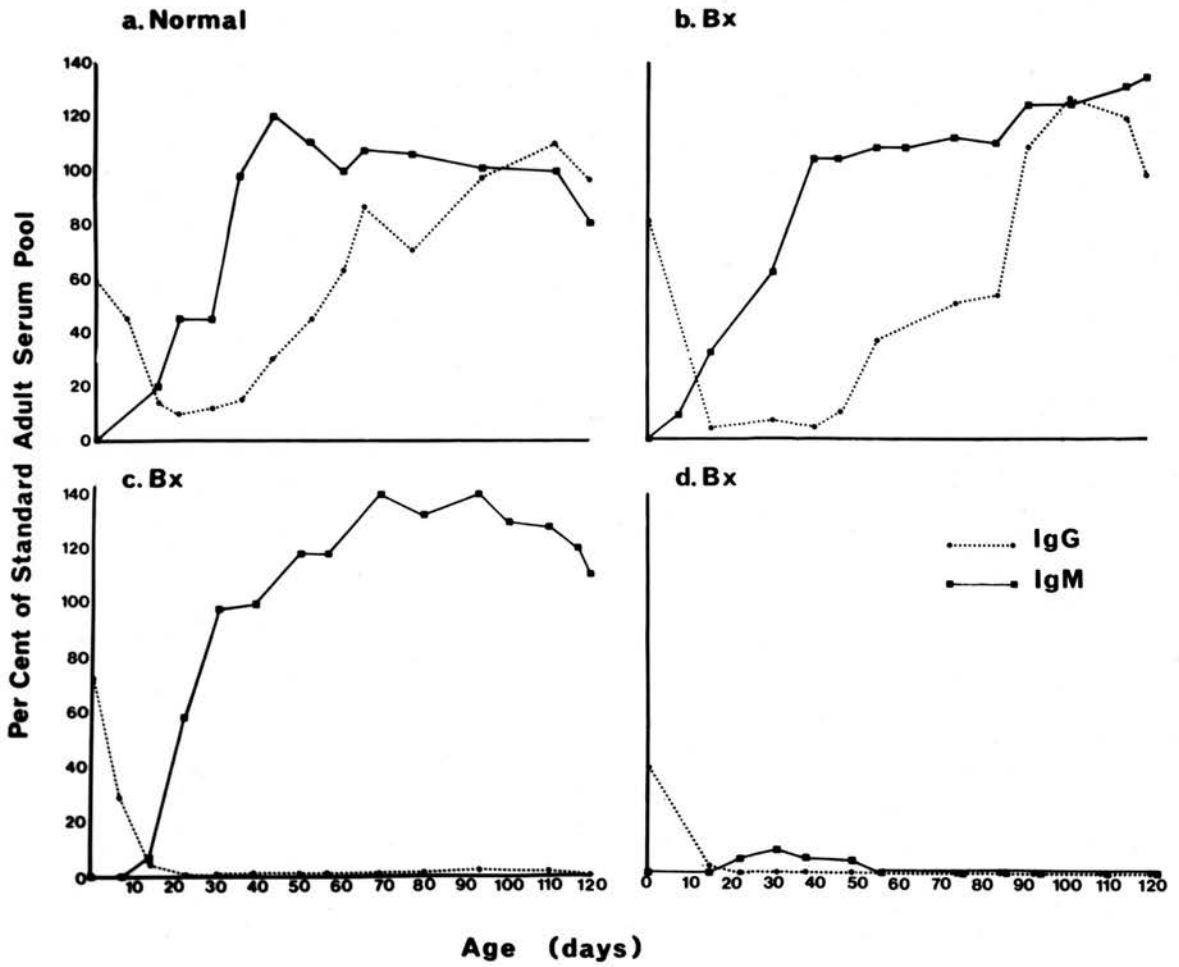
The remaining bursectomised birds were deficient in both IgG and IgM and were often totally agammaglobulinaemic (Fig. 29d).

Although some birds had normal IgM levels with an absence of IgG, none had normal IgG levels without IgM.

Similar findings have been reported in hormonally

Figure 29

**Development of Immunoglobulin Synthesis in Normal
and Bursectomised Chicks**



bursectomised birds by Warner et al., (1969), although their measurements were by immunoelectrophoresis and therefore not strictly quantitative.

Cooper et al., (1969) also found that all embryonically bursectomised birds lacking IgM were deficient in IgG, but not vice-versa. In contrast with the present results, all their birds were deficient in IgG. However, the IgG levels were measured at 44 days of age, at which time in the present experiment even those birds which eventually developed normal IgG levels showed a deficiency.

These authors also found that proportionally more chickens bursectomised 4 days before hatching had no detectable IgM, than chickens bursectomised 3, 2 or 1 day before hatching. They interpreted these results as indicating that the 17th day of embryonic development was the critical time after which IgM synthesis was not inhibited by bursectomy. However, individual agammaglobulinaemic birds were found in the 18 and 19 day bursectomised groups, and as already mentioned, the numbers of birds included in the study were small.

In the present study, individual birds showing the three different patterns of immunoglobulin synthesis were found in all three bursectomised groups, and it was not possible to demonstrate that the results for the 17 day bursectomised group were different from the other groups. The hatching rate of chickens bursectomised on

day 17 had, however, been poor, and the operation had proved more difficult at this time with a greater risk of rupturing the bursa. Consequently another much larger group of chickens was bursectomised on day 17, and the serum IgG and IgM levels of the individual birds at 4 months of age were compared with those of 18 and 19 day bursectomised and control chickens of the same age. With more practice the operation on 17 day old embryos became as successful as on older embryos. Altogether the sera from 77 birds were tested - 17 controls, 15 bursectomised on day 19, 17 on day 18 and 28 on day 17.

The results of this experiment are shown in Fig. 30. This shows clearly that the degree of inhibition of IgM synthesis is greater in chickens bursectomised on the 17th day of incubation than on the 18th or 19th day. IgG synthesis is effectively inhibited in all the groups.

Statistical analysis of these results comparing the 17 day group with the 18 day + 19 day groups is shown in Table 6. This indicates that there is a definite difference between the 17 day and the other groups in the numbers of birds with IgM levels falling below 20% of the standard adult pool. The probability is less than 0.1 and would almost certainly reach the 5% level if a larger number of birds was tested. There was no difference between the 18 and the 19 day groups.

These results along with recent evidence described

Figure 30

THE EFFECT OF BURSECTOMY AT DIFFERENT TIMES DURING INCUBATION

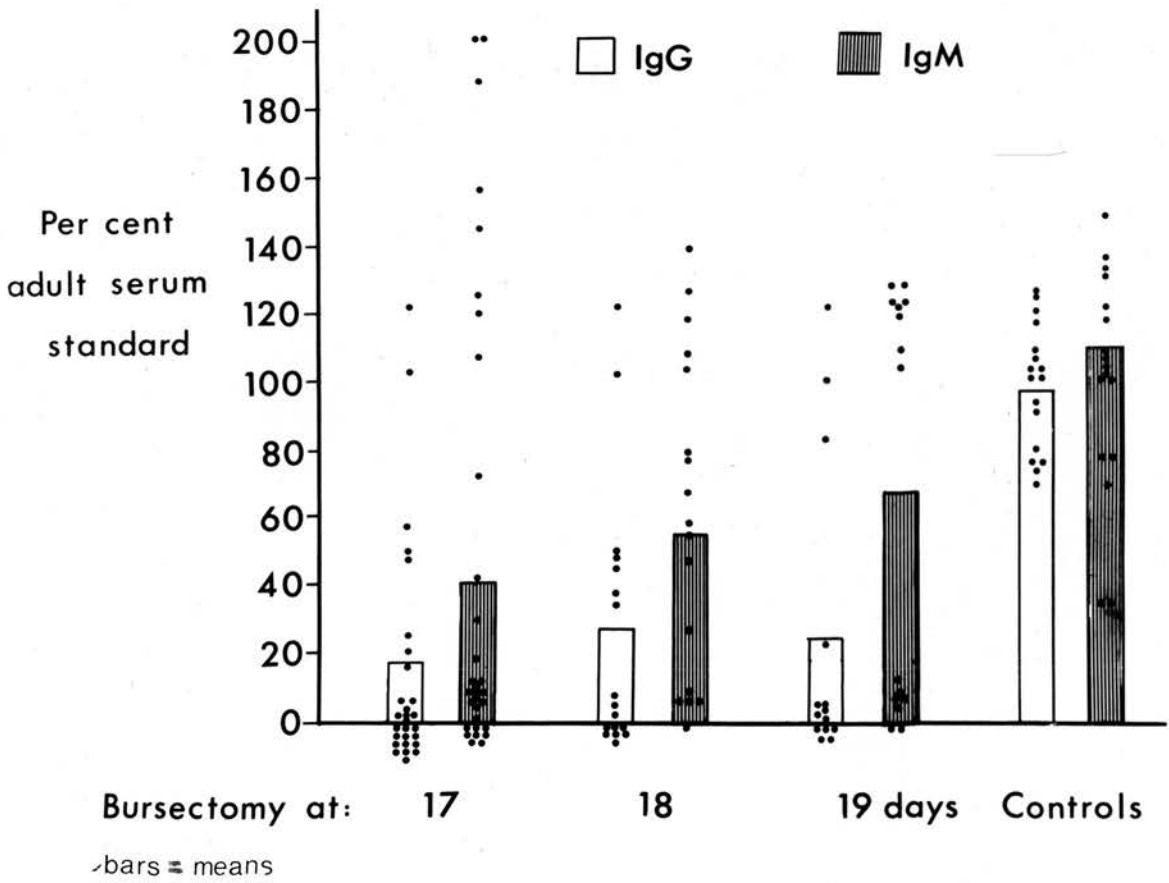


Table 6 : Statistical analysis by a $2 \times 2 \chi^2$ table comparing the numbers of birds in the 17 day bursectomised group with the numbers in the 18 day + 19 day groups, whose serum IgM levels at 4 months of age fell above and below 20% of the standard adult pool.

| | 17 day bursectomised | | 18+19 day bursectomised | | Total |
|-----------|----------------------|------------|-------------------------|------------|-------|
| | observed | : expected | observed | : expected | |
| Above 20% | 10 | : 14 | 20 | : 16 | 30 |
| Below 20% | 18 | : 14 | 12 | : 16 | 30 |
| Total | 28 | : 28 | 32 | : 32 | 60 |

Using Yate's correction for continuity:

$$\chi^2_{(1)} = \sum \frac{(|O-E| - 0.5)^2}{E}$$

$$= 3.28$$

From the tables for χ^2 with 1 degree of freedom the 5% point is 3.84 and the 10% point 2.71.

Therefore $0.1 > P > 0.05$

below in the discussion indicate that the bursa exerts its influence on IgM synthesis but not IgG synthesis before the 17th day of embryonic development. The fact that birds bursectomised as late as day 19 can be rendered agammaglobulinaemic is not incompatible with this basic concept since the rate of development of different embryos is never identical. There can also be a difference of as much as 24 hours actual incubation time between eggs placed in an incubator simultaneously, because of the variability in the time an egg may lie in the hen's oviduct before being laid.

Histology

Histological examination of the spleen, Harderian gland and caecal tonsils of most of the bursectomised birds revealed severe lymphocyte depletion and a scarcity or total absence of germinal centres and plasma cells. However, some bursectomised chicks showed severe depletion of lymphoid tissue only in the spleen with normal Harderian gland and caecal tonsil, or a normal histological structure of all three organs. In most cases the thymus was normal. The histological findings are recorded in Table 7.

Maternally derived IgG

One interesting finding in the study of the ontogeny of IgG synthesis in chickens, was that the IgG found in day old chicken serum, i.e. maternally derived IgG differed electrophoretically from the IgG actively synthesised by the chicken itself. Fig. 31 shows an immunoelectrophoresis

Table 7: Histological examination of spleen, caecal tonsil, and Harderian gland from bursctomised chickens.

| Chicken No. | Age at Bursectomy | Ig. levels | | Histology | | |
|-------------|-------------------|------------|-----|--|--|------------------------|
| | | IgG | IgM | Spleen | Caecal tonsil | Harderian gland |
| 164 | 19 days | - | ++ | No follicles or plasma cells. Lymphocyte depletion. | No follicles or plasma cells. Lymphocyte depletion. | No plasma cells. |
| 166 | 19 days | - | - | No follicles or plasma cells. Lymphocyte depletion. | No follicles or plasma cells. Lymphocyte depletion. | No plasma cells. |
| R 3678 | 17 days | - | + | Lymphocyte depletion. | Lymphocyte depletion. | |
| B 176 | 17 days | - | - | No follicles or plasma cells. Lymphocyte depletion. | | |
| B 177 | 17 days | + | + | No follicles. Lymphocyte depletion. | Lymphocyte depletion. | |
| B 215 | 17 days | + | ++ | Lymphocyte depletion. | Necrosis. | |
| B 218 | 17 days | + | ++ | No follicles. Lymphocyte depletion. | Necrosis. | |
| B 179 | 17 days | NT | NT | Lymphocyte depletion. | Lymphocyte depletion. | Very few plasma cells. |
| B 189 | 17 days | - | - | Lymphocyte depletion. | Lymphocyte depletion. | |
| B 190 | 17 days | NT | NT | Lymphocyte depletion. | Complete absence of lymphoid tissue. | |
| B 202 | 17 days | - | - | Lymphocyte depletion. | Lymphocyte depletion. | |
| B 221 | 17 days | ++ | ++ | Lymphocyte depletion. | Lymphocyte depletion. | |
| R 3680 | 17 days | - | - | Lymphocyte depletion. | Lymphocyte depletion. | |
| B 178 | 17 days | NT | NT | Severe lymphocyte depletion. | Lymphocyte depletion. | Normal. |
| B 180 | 17 days | - | + | Lymphocyte depletion. | Normal. | |
| B 184 | 17 days | NT | NT | Normal. | Normal. | Normal. |
| R 3679 | 17 days | NT | NT | Normal. | Normal. | Few plasma cells |

which illustrates this difference. No antigenic difference has been shown between the two types of IgG by double diffusion studies.



Figure 31.

Immunoelectrophoresis slide showing difference in electrophoretic mobility between maternally derived IgG in day old chicken serum and IgG in adult chicken serum.

DISCUSSION

The percentage of birds rendered deficient in serum immunoglobulins by bursectomy carried out surgically on the 17th day of incubation compare favourable with results obtained by hormonal bursectomy, but are rather lower than those reported by Van Meter et al., (1969) for bursectomised-irradiated chickens. At 8 weeks of age 59% of their birds had IgG levels below 2% of the normal adult pool, and no detectable IgM, whereas in the present experiment 46% of embryonically bursectomised chickens had no detectable IgG, and 29% had no detectable IgM. However, the regular finding of complete agammaglobulinaemia in 29% of operated birds, demonstrates that the method of surgical bursectomy in ovo is a useful and practical method to employ for further experiments, especially when its complete specificity is taken into account.

These results further confirm that the bursa of Fabricius is the only organ capable of effecting the differentiation of lymphoid stem cells along the plasma cell line to the stage of being capable of synthesis of either IgM or IgG. This conclusion was also reached by Warner et al., (1969) from results in hormonally bursectomised chicks, by Cooper et al., (1965) from bursectomy-irradiation experiments, and by Cooper et al., (1969) from results of embryonic bursectomy. In the face of this quite conclusive evidence, the doubts expressed by Jankovic and Isakovic as recently as 1967

about the absolute dependence of antibody production on the bursa seem groundless.

The present results have also confirmed the sequential nature of the differentiation process, so that removal of the bursa at 4 or more days before hatching can prevent the development of the ability to synthesise both IgM and IgG, whereas its removal less than 4 days before hatching prevents only the development of IgG synthesis. There have been two different suggestions put forward for the possible mechanism involved in this sequential differentiation process (Van Alten et al., 1968; Cooper et al., 1969).

The first is that the effect is a quantitative one, the earlier the bursectomy is carried out, the fewer differentiated cells will have seeded from the bursa to the peripheral tissues. If the bursa is removed early enough no competent cells will have left the bursa, but if the bursa is removed after only a limited number have seeded, then these few cells might be quickly saturated by antigens, and differentiate to produce IgM antibodies, the saturation suppressing the cell division necessary, according to Sterzl, (1966) and Sterzl and Silverstein, (1967), for IgG antibody synthesis. In support of this possibility Cooper et al., (1969) have quoted the theory presented by Baglioni, (1963) for the change-over from production of foetal haemoglobin to adult haemoglobin - that is that cell division is necessary for derepression of the ability to synthesise the adult type,

and that in foetal life or in cases of anaemia, where the demand for differentiation is highest, insufficient cell division may occur for the derepression mechanism to operate. However, if, as at present it is generally believed, the clonal selection theory of predetermined specificities of antibody producing cells holds true, then it seems unlikely that all the seeded plasma cell precursors of random antigen specificities should simultaneously be saturated with antigen. The range of different specificities would be more likely to be reduced. This would lead to an inability to mount antibody responses after specific antigenic challenge rather than an abolition of total IgG production. This seems to be the case, since more chickens show severe deficiencies in specific antibody responses than in serum immunoglobulin levels. Experiments to be described in the next section where antibody responses are compared with serum immunoglobulin levels, illustrate this fact. Also against this particular theory is the fact that the incidence of dysgammaglobulinaemia was not increased in bursectomised birds by the administration of large doses of antigen immediately after hatching (Van Alten et al., 1968), and results of experiments by Ivanyi et al., (1966) where IgM synthesis was actually inhibited in chickens by increasing the dose of antigen.

The second alternative is that the length of time spent by a cell inside the microenvironment of the bursa

determines its differentiation, cells residing there for longer periods becoming differentiated to IgG synthesis, or else that the microenvironment of the bursa changes in some way about the 17th day of incubation, and before this time differentiation to IgM synthesis is induced, whereas later differentiation to IgG synthesis occurs. The present evidence of the 17th day being a critical stage would favour this alternative. Also in support of this particular theory is the finding that bursal lymphoid cells change in respect to their nucleic acid ratios at different stages in embryonic life (Cooper et al., 1967) and that bursal tissue from 19 day old embryos could produce only 19S immunoglobulins in culture, whereas bursal tissue from older birds could produce both 19S and 7S immunoglobulins

The strongest evidence in favour of this alternative, however, is provided by recent work by Kincade and Cooper, (1971). They have shown by fluorescent antibody studies that cells containing μ and light chains are present in bursal follicles as early as the 14th day of incubation, and can be observed in other sites outside the bursa by day 17. γ chains, on the other hand, are not seen inside the bursa until the 21st day of incubation, or outside the bursa until 4 days after hatching.

Thus, in the light of the available evidence, the most attractive theory is that stem cells enter the bursa and start to differentiate immediately to a plasma cell

line capable of IgM production. Some of these cells start to migrate at once to the peripheral lymphoid tissues. After a longer period of time inside the bursa, cells, probably of the same line, differentiate further to be capable of IgG production and these cells do not seed to other sites until around hatching time. There is evidence that it is the same line of cells which changes from IgM to IgG production during differentiation inside the bursa, for many cells containing both μ and δ chains were observed in the bursa by Kincade and Cooper, whereas such cells were very rarely seen elsewhere in the body.

PART III

THE LONG TERM EFFECT OF BURSECTOMY
ON IMMUNOGLOBULIN SYNTHESIS

INTRODUCTION

One aspect of plasma cell differentiation and immunoglobulin production which is not at present clear, is whether the competent cells which have seeded from the bursa (the "X cells" of Sercarz and Coons (1962)) are a self-replenishing pool, or whether the entire population of these competent cells needed to function throughout the total life-span, is derived directly from the bursa during the months before the organ atrophies and disappears, and is not capable of multiplication without further differentiation to end cells.

Cooper et al., (1969) suggest that these cells are capable of proliferation and differentiation in the absence of the bursa, because chickens bursectomised at hatching can mount normal secondary antibody responses, synthesise normal amounts of IgG and IgM, and have normal numbers of plasma cells in their tissues.

However these measurements have mostly been taken within a fairly short period of time after hatching, and if, as is reasonable to suppose, competent cells in excess of normal requirements are seeded from the bursa, one would expect that a deficiency would not become apparent for a considerable period.

On the other hand, with the number of seeded competent

cells severely reduced due to removal of the bursa before hatching, a deficiency might be expected to develop quite soon in birds which initially synthesised substantial quantities of either IgM alone or both IgG and IgM.

Although Van Meter et al., (1969) reported that neonatally bursectomised chicks developed higher than normal levels of circulating immunoglobulins which persisted throughout the 13 week experimental period, experiments by Arnason and Jankovic, (1967) continued over 6 months, indicated that bursectomised birds began at that stage to develop an IgG deficiency although IgM levels remained normal.

In order to investigate this aspect further, a group of 12 chickens bursectomised on the 17th day of incubation, and 12 control chickens, were studied over a period of 8 months. The birds were kept for this long-term study alone and were not given any antigenic stimuli. They were sampled regularly at 1 week and then 1 month intervals over the experimental period and their IgG and IgM levels measured.

RESULTS

The graph of the mean IgG and IgM levels over the test period of the bursectomised and control chickens is shown in Fig. 32. Most of the bursectomised group at first produced substantial levels of IgM or both IgG and IgM. From 4 months onwards, however, the mean IgG and IgM for the bursectomised birds began to fall. The

SERUM Ig LEVELS FROM HATCHING

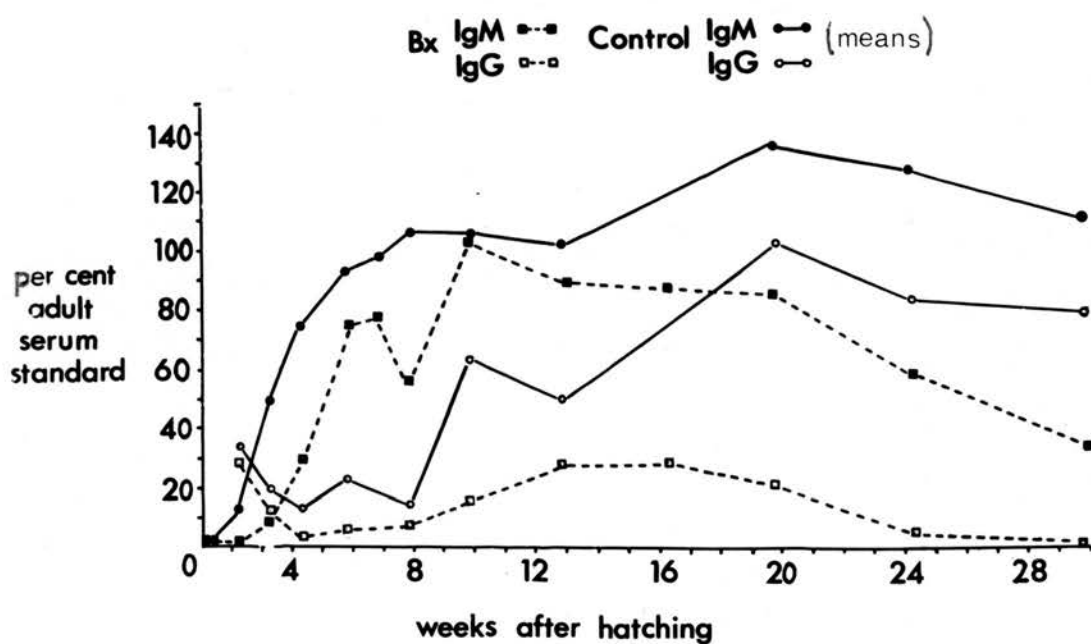


Figure 32.

Long-term effect of bursectomy on
 immunoglobulin levels.
 Mean IgG and IgM levels for bursectomised
 and control chickens over 8 month period.

decline continued until the end of the test period of $7\frac{1}{2}$ months by which time the mean IgG had reached less than 1% and the IgM about 30% of the standard adult pool. The general trend of increasing immunoglobulin deficiency with age was demonstrable in spite of large variations in immunoglobulin levels between individuals.

The eventual decline to agammaglobulinaemia in bursectomised birds over a long period was even more apparent when individual immunoglobulin levels were plotted for 7 17 day bursectomised birds which survived beyond 30 weeks of age (Fig. 33). All these birds became completely deficient in IgG, and most, in fact, developed a total agammaglobulinaemia. Only one of the 7 maintained normal IgM production.

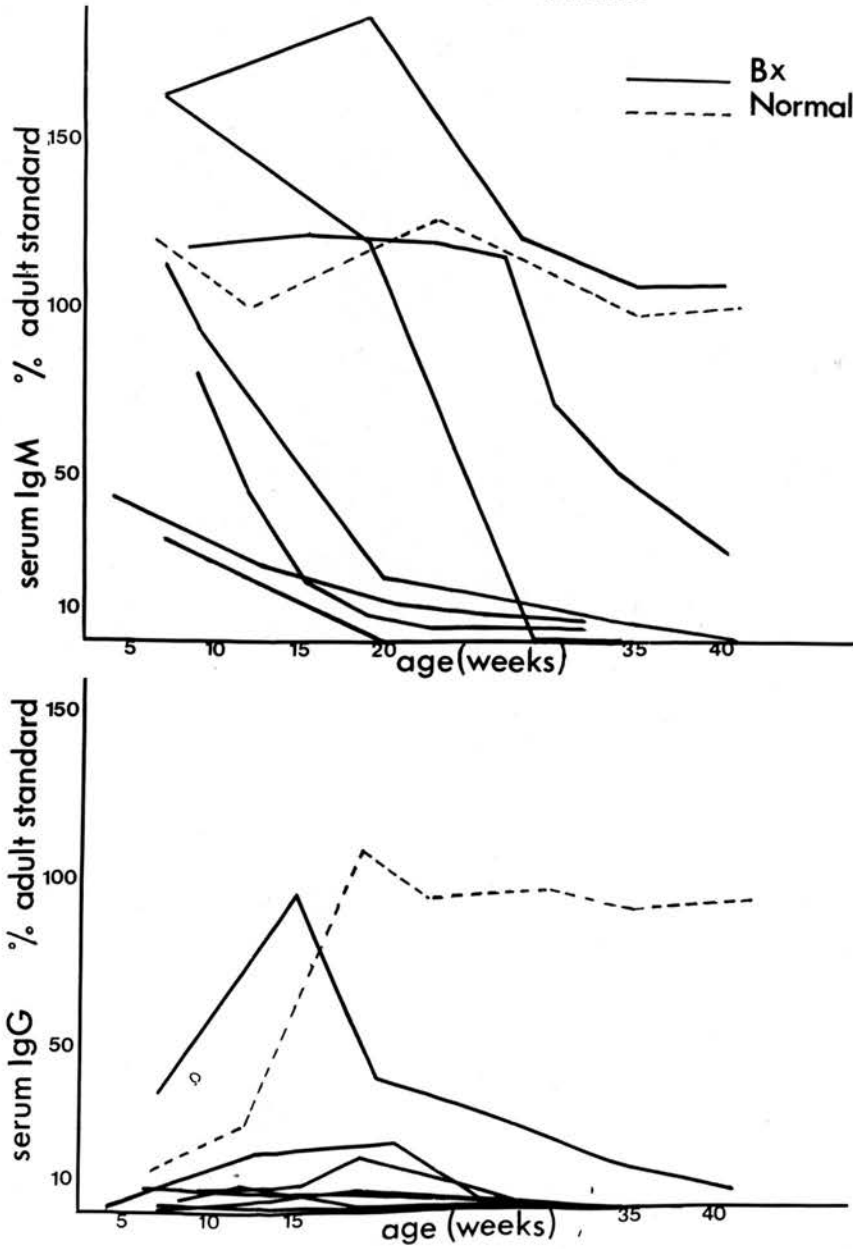
DISCUSSION

These results suggest that the immunocompetent cells which seed from the bursa to the peripheral lymphoid tissues before bursectomy, differentiate without further bursal influence to "end cells" which secrete immunoglobulin, but that they are not self replenishing.

If replication of these immunocompetent precursor cells does occur it must, therefore, be on a very limited scale.

Figure 33

SERUM IMMUNOGLOBULIN LEVELS IN Bx BIRDS
SURVIVING BEYOND 30 WEEKS



lines represent individual birds

PART IV

THE EFFECT OF BURSECTOMY ON 7S γ 1 SYNTHESIS

The results of experiments reported in Section II which were designed to show whether or not the 7S γ 1 protein was an immunoglobulin suggested that it was not, but failed to provide definite proof. It was hoped that further evidence would be provided by a study of the effect of bursectomy on 7S γ 1 synthesis.

Serum from 9 bursectomised birds, of which 3 had no detectable IgG or IgM, 3 had only IgM, and 3 had both IgG and IgM, were tested by immunoelectrophoresis using anti-7S γ 1 antiserum in the troughs. All samples contained 7S γ 1, the lines being identical to those obtained with normal chicken serum.

Serum 7S γ 1 levels from hatching to 16 weeks of age were also plotted along with IgG and IgM levels of normal and bursectomised birds representative of the three patterns of immunoglobulin development already described (Fig. 34). The pattern of 7S γ 1 synthesis did not vary markedly between the birds, and showed no correlation with either IgG or IgM synthesis. The levels in all birds fluctuated markedly throughout the test period. Individual serum 7S γ 1 levels at 3 months of age are shown in Fig. 35 along with IgG and IgM levels in bursectomised birds, arranged in 3 groups according to immunoglobulin status. Again there appeared to be no correlation between 7S γ 1

Figure 34

**7S γ 1 Synthesis in Normal and Dysgammaglobulinaemic
Bursectomised Chicks**

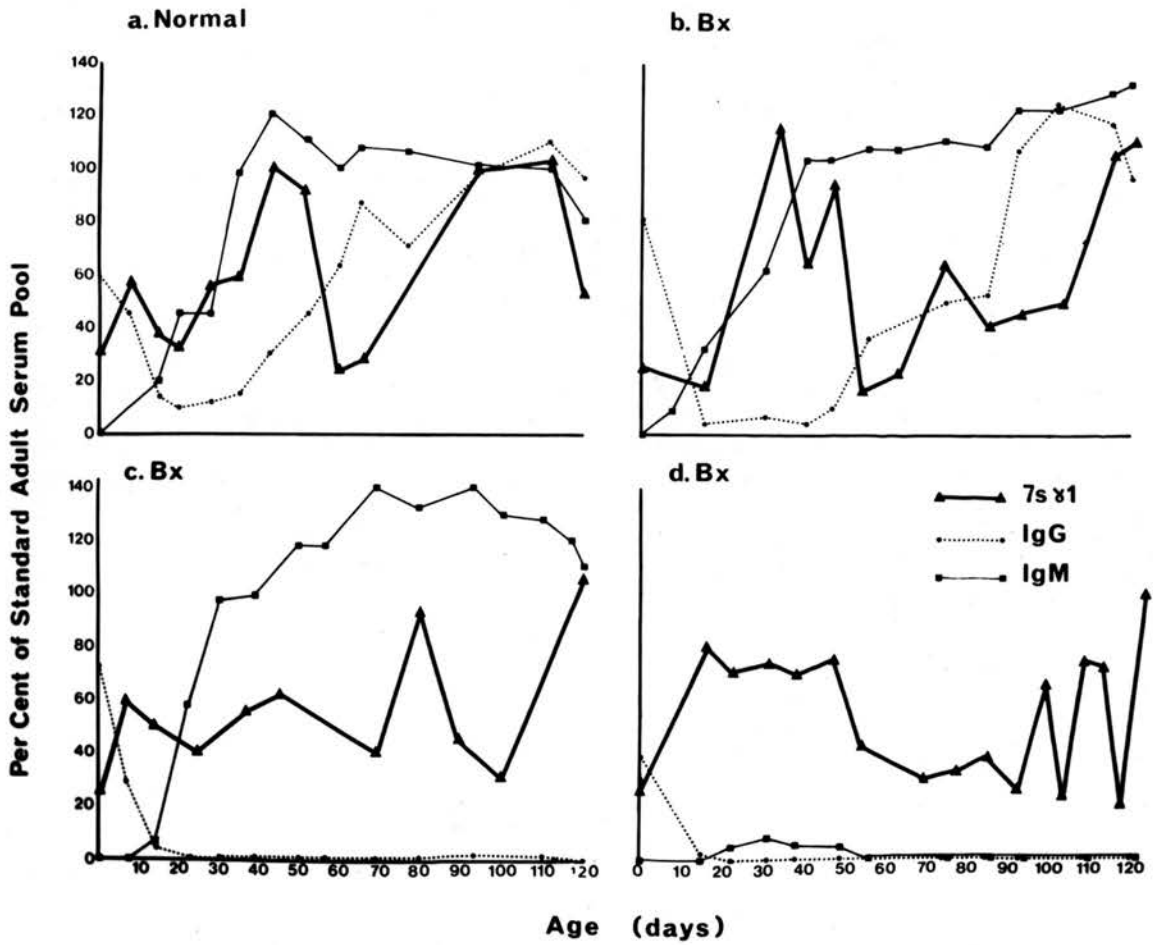
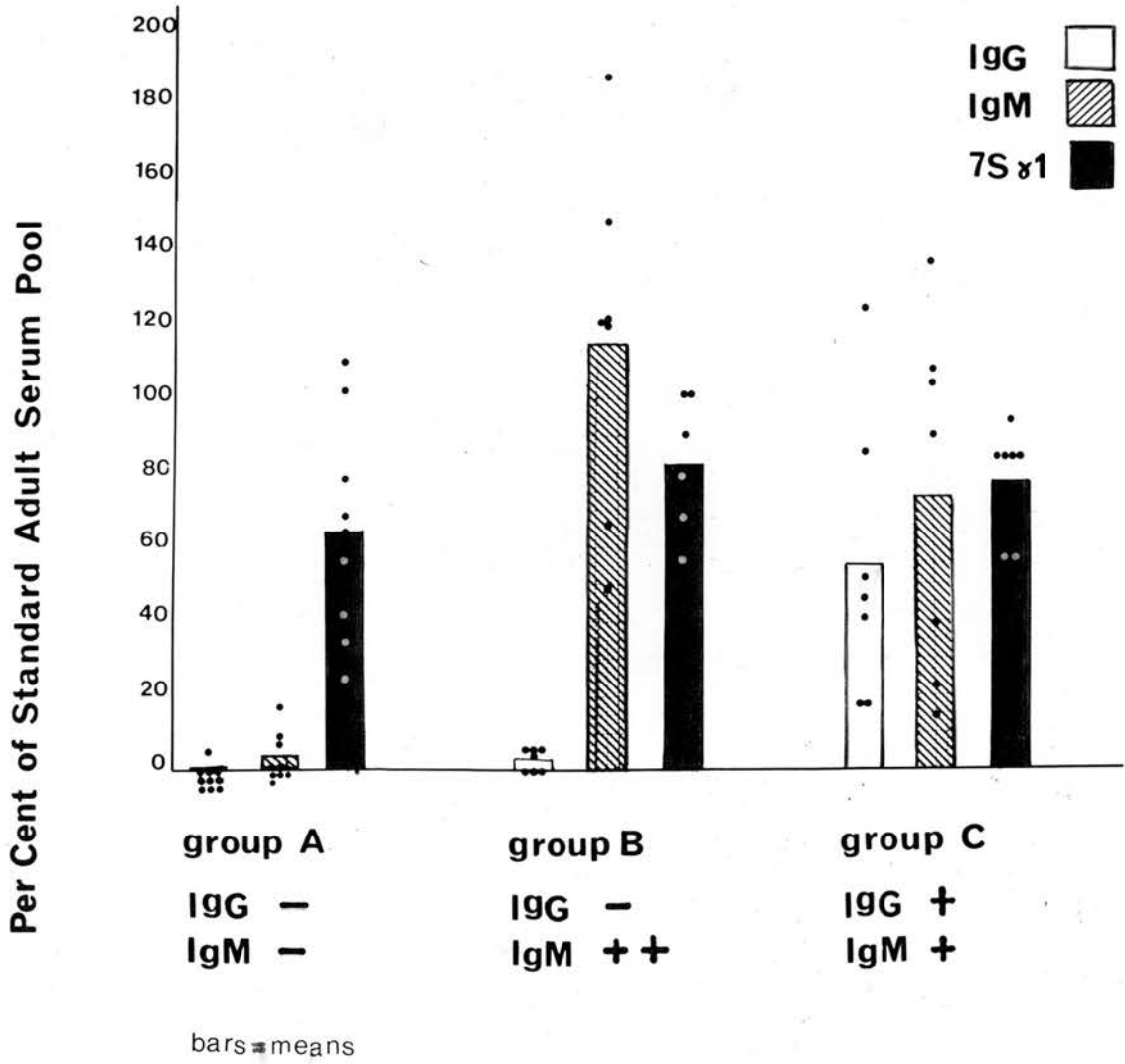


Figure 35

7S κ 1 Synthesis in Dysgammaglobulinaemic Bursectomised Chicks



levels and the two immunoglobulins.

These results indicate that 7S γ 1 synthesis is either not under the control of the bursa of Fabricius, or else the bursa must exert its influence before the 17th day of embryonic development. This evidence, along with the results of the experiments reported in Section II would strongly suggest that this protein is not an immunoglobulin.

SECTION IV

**STUDIES ON IMMUNOGLOBULIN FUNCTION:
IMMUNOGLOBULIN SYNTHESIS IN RELATION TO SURVIVAL,
AND SPECIFIC ANTIBODY RESPONSES.**

PART I
SURVIVAL

INTRODUCTION

In human medicine the various agammaglobulinaemia and hypogammaglobulinaemia syndromes have been extensively reported and are reviewed by Good et al., (1962b). If not given large doses of gamma globulin, patients with the Bruton form of agammaglobulinaemia, which closely resembles the deficiency produced by bursectomy, are plagued by recurrent, severe, life-threatening bacterial infections. These occur one after another in a succession of attacks, dominated by pneumonia, meningitis, pyoderma, septicaemia, otitis, sinusitis, and conjunctivitis. Even with large monthly doses of gamma globulin and modern antibiotic therapy, many patients die. From this, one would expect a similar clinical picture in bursectomised agammaglobulinaemic chickens, and it is of interest to study the survival of these birds.

Van Alten et al., (1968) studied survival rates in chickens bursectomised at hatching or as 19 day old embryos, and noted a sharp decline in survival which started about the 6th week of life. By 8 weeks after hatching only 10% of embryonically bursectomised birds, and 20% of neonatally bursectomised birds were still alive, as compared with 65% of normal birds and over 80% of sham operated controls.

In the course of the present work, over 100 chickens have been bursectomised as embryos and a study has been made of their survival.

EXPERIMENTAL OBSERVATIONS

The mortality rate for these bursectomised birds is shown graphically in Fig. 36. The birds were divided into two batches. Batch 1 includes 17, 18 and 19 day embryonically bursectomised birds whose serum immunoglobulin synthesis was studied over a 20 week period, at the end of which survivors were killed for post mortem examination. Batch 2 consists of 17 day embryonically bursectomised birds which were used for long-term studies, antigen studies etc. None of batch 2 were killed unless they were severely ill.

From the graph and Table 8 it can be seen that the total mortality and also the pattern of mortality differed between the bursectomised and the control groups. 87.8% of bursectomised chicks died during the 33 week experimental period, whereas only 43.2% of control birds died during this time. Most of the deaths in the bursectomised group (44.8%) occurred during the first 7 weeks of life and were due mainly to E.coli septicaemia, whilst few control chickens died at this age. The vast majority of deaths in the control group occurred at between 7 and 20 weeks of age, 79.3% as compared with only 22.4% for the bursectomised groups. Thereafter the numbers of bursectomised birds declined steadily whereas very few deaths (10.3%) occurred among the normal birds from 20 weeks onwards.

In an attempt to discover whether serum immunoglobulin

Figure 36

SURVIVAL RATES IN NORMAL AND BURSECTOMISED BIRDS

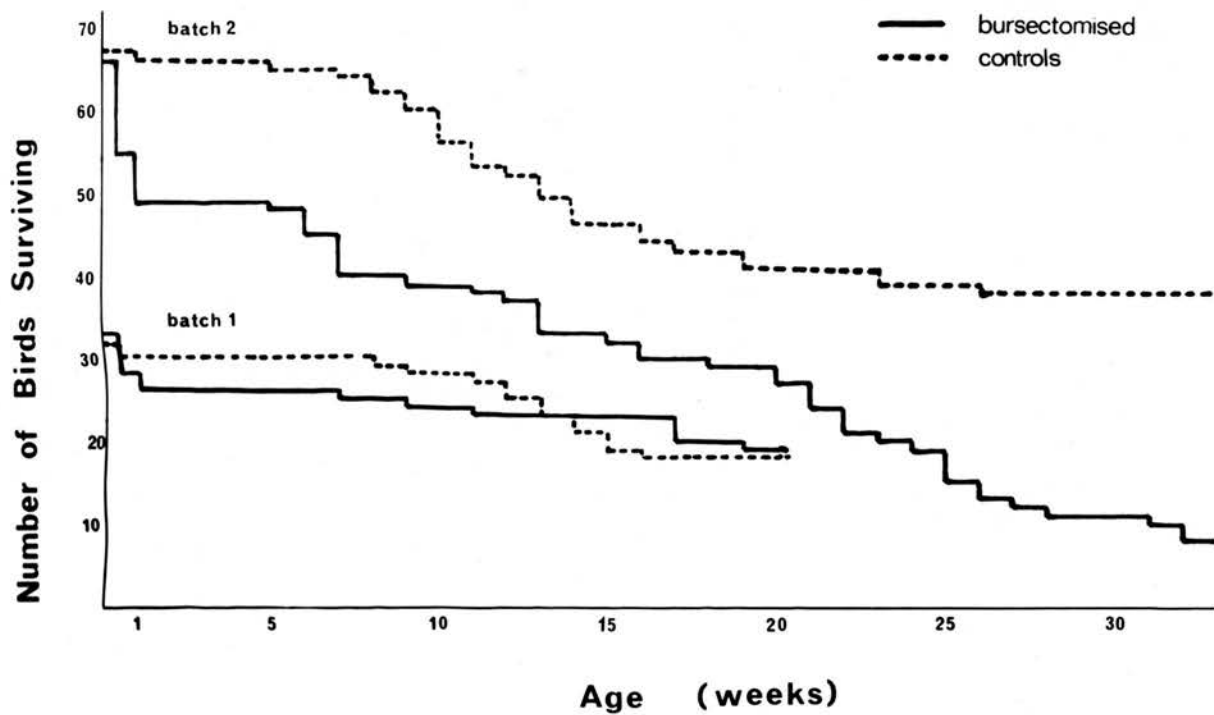


Table 8a : Mortality in normal and bursectomised chickens.

| | Percentage of birds which died during experimental period | Percentage deaths which occurred during undernoted periods | | |
|---------------|---|--|------------|------------------|
| | | 0-7 weeks | 7-20 weeks | 20 weeks onwards |
| Normal | 43.2 | 10.3 | 79.3 | 10.3 |
| Bursectomised | 87.8 | 44.8 | 22.4 | 32.7 |

Table 8b: Survival of bursectomised birds in relation to serum immunoglobulin levels.

| Serum Immunoglobulin Levels | Number of birds in group | Number surviving beyond 32 weeks | Average survival time (after 6 weeks) of remainder |
|-----------------------------|--------------------------|----------------------------------|--|
| Group 1 IgG - IgM - | 16 | 0 | 11.4 weeks |
| Group 2 IgG - IgM ++ | 13 | 1 | 16 weeks |
| Group 3 IgG + IgM + | 13 | 2 | 12.2 weeks |

levels had any effect on survival, mortality rates were plotted for three groups of bursectomised birds; those whose serum contained both IgG and IgM, those with no IgG but high levels of IgM, and those which were deficient in both immunoglobulins (Fig. 37).

From the average survival times shown in Table 8b it can be seen that there was no significant difference between any of the groups. The ability to synthesise immunoglobulins did not confer any advantage on the bursectomised birds which did so, as compared with agammaglobulinaemic birds.

DISCUSSION

The reason for the sharp decline in survival in normal birds as compared with bursectomised birds during the 7 - 20 week period is not certain. However, although histological examination of the sciatic nerves and /or ovaries of casualties revealed Marek's disease lesions in both control and bursectomised birds, the classical paralysis symptoms were almost exclusively confined to the control group (Table 9). The post mortem finding of tumours in the gonads, liver and kidneys was also common in the control groups but rare in bursectomised birds. Since Marek's disease symptoms usually appear from the 6th week of life onwards, it is suggested that the rapid decline in control birds in the 7 - 20 week period was probably due to this disease. In the

Figure 37

**SURVIVAL RATES IN NORMAL AND BURSECTOMISED BIRDS
IN RELATION TO IMMUNOGLOBULIN STATUS**

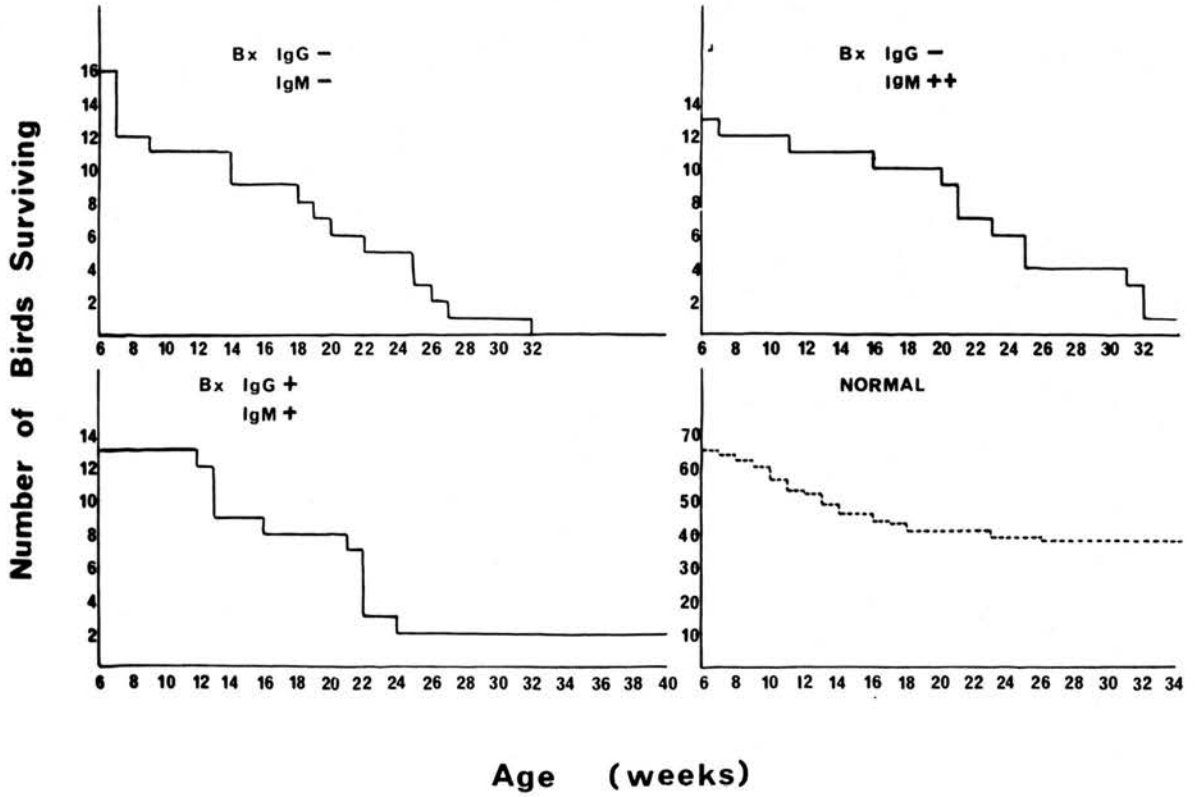


Table 9 : Histological signs of Marek's disease in Normal and Bursectomised chickens.

| Chicken Number | Treatment | Marek's disease lesions in | |
|----------------|-----------|----------------------------|-----------------------|
| | | Sciatic nerve | Ovary |
| 1 | Control | + | |
| 2 | Control | | + |
| 3 | Control | - | + |
| A 229 | Control | + slight | |
| B 235 | Control | - | |
| B 243 | Control | - | |
| B 245 | Control | + severe | + |
| R 3694 | Control | - | |
| B 247 | Control | - | + |
| R 3660 | Control | + | |
| R 3661 | Control | + slight | |
| R 3693 | Control | + | + |
| R 3695 | Control | - | |
| R 3696 | Control | - | |
| 395 | Control | - | + (possibly leucosis) |
| R 3866 | Control | - | |
| B 211 | Control | - | + (possibly leucosis) |
| R 3678 | BX | - | |
| B 170 | BX | + severe | |
| B 176 | BX | - | |
| B 215 | BX | - | |
| B 218 | BX | - | |
| R 3679 | BX | - | |
| B 178 | BX | - | |
| B 179 | BX | - | |
| B 221 | BX | + | |
| B 193 | BX | - | |
| R 3680 | BX | - | |
| B 208 | BX | - | |
| B 241 | BX | - | |
| B 244 | BX | - | |

In the bursectomised birds, although a few were shown by histological examination to be infected, the disease may not have been acute enough to cause this rapid mortality.

The development of Avian Leucosis (Visceral lymphomatosis) has been shown to be prevented by surgical bursectomy at either 1 day or 29 days of age (Peterson et al., 1964). Recent reports by Payne and Rennie, (1970) and Fernando and Calnek, (1971), however, on the influence of the bursa on Marek's disease have indicated that this disease is not bursa dependent.

The lack of correlation between serum immunoglobulin levels and average survival time is not surprising since, as will be shown in Part II, severe deficiencies in specific antibody responsiveness can exist in birds with normal serum immunoglobulin levels.

Although bursectomy does cause a considerable survival disadvantage, on the whole the dramatic clinical picture of recurrent infection particularly at the body surfaces - pyoderma, pneumonia, sinusitis, etc. - was not seen in the bursectomised birds. Most of these birds died from septicaemia without obvious prior infections of the type mentioned.

It would appear therefore, that in the fowl the protective role of specific humoral immunity is of less importance than in man. A further possibility is that the bursa does not control all immunoglobulin synthesis. For example, immunoglobulin of surface secretions

(IgA analogue?) may be bursa independent. Certainly, it has been shown that the resistance of the fowl to invasion by its intestinal microflora is not abolished by bursectomy (Fuller and Jayne-Williams, 1970).

PART II

SPECIFIC ANTIBODY RESPONSES IN RELATION TO SERUM
IMMUNOGLOBULIN SYNTHESIS IN BURSECTOMISED BIRDS

INTRODUCTION

The inhibiting effect of all the various methods of bursectomy on antibody responses has been extensively reported in the literature and has already been discussed in previous sections. Briefly, bursectomy performed up to 5 weeks after hatching can cause an inhibition in primary antibody responses to a variety of different antigens (Glick et al., 1956; Mueller et al., 1960, 1962), the earlier the operation is performed the more marked the inhibition. However, in most birds bursectomised even as early as the day of hatching, secondary responses can approach normal levels and serum immunoglobulin production is also normal (Claflin et al., 1966; Jankovic and Isakovic, 1966, 1967; Arnason and Jankovic, 1967). Neonatal bursectomy and irradiation, and hormonal bursectomy can totally eliminate both primary and secondary responses to several antigens in a proportion of birds so treated, and many of these birds also show marked immunoglobulin deficiencies (Cooper et al., 1966a; Warner et al., 1969).

A direct correlation between the degree of inhibition of serum immunoglobulin synthesis and of specific antibody responses in individual birds has not, however, been demonstrated. Cain et al., (1969) compared serum IgG

and IgM antibody responses to sheep red cells in chickens bursectomised as embryos. Although they showed a correlation between time of bursectomy before hatching and antibody titres, they failed to show a similar correlation between immunoglobulin levels and antibody titres, and strangely several birds with very low serum IgG or IgM levels produced fairly high amounts of IgG or IgM antibodies.

Warner et al., (1969) working with hormonally bursectomised birds, demonstrated deficiencies in antibody responses to sheep red cells, DNP, Brucella, and bacteriophage in some birds which had substantial levels of either IgM alone or IgG and IgM, although in the main those which had both IgG and IgM developed antibody titres (mainly mercapto-ethanol sensitive), whilst those which were agammaglobulinaemic did not.

The experiments reported in this section were designed to study antibody responses to several different antigens in relation to immunoglobulin levels in chickens bursectomised 4 days before hatching. It was hoped that the results might shed some light on the still very much unresolved problem of the mechanisms of development of specific antibody responses, and the specific role of the normal serum immunoglobulins.

MATERIALS AND METHODS

Chickens

Chickens bursectomised surgically as 17 day old embryos were used for BSA and sheep red cell inoculations. For the S.gallinarum experiment chickens which had been surgically bursectomised on the day of hatching and X-irradiated the following day were used. This method of bursectomy was adopted because of a period of low hatchability in the flock which provided the fertile eggs. During this period it proved impossible to obtain even moderate hatching rates using embryonic bursectomy, and so, rather than change the strain of chickens used, it was decided to temporarily adopt neonatal bursectomy plus irradiation. Halothane anaesthesia was used and the incisions were closed with nylon sutures which were removed 1 week later.

The details of the X-irradiation procedure are as follows:

Dose - 61.09 r/min. (backscatter approx. $1\frac{1}{2}$

Focus Skin Distance (FSD) - 75 cm.

Total time - 10 minutes 40 seconds (5 mins. 20 secs. each side).

Filter - 0.5 mm. copper + 1 mm. aluminium \equiv Half Value Layer of 1.2 mm. copper.

230 KV.

15 ma.

The chickens were packed tightly into a small plastic

container, and any free spaces were filled with wax blocks. Control chickens were given the same dose of irradiation as bursectomised birds.

All birds used were over 8 weeks of age at the time of antigen administration.

Antigens and Inoculation Procedures

The antigens used and their administration were as follows:

BSA - Bovine serum albumin (Armour Pharmaceutical Company Ltd.) was injected intravenously at a dose of 20 mg./Kg. body weight.

Sheep red blood cells - These were washed 6 times in sterile 0.85% saline, and injected intravenously as a 10% suspension in sterile saline. The dose was 0.5 ml./Kg. body weight.

Salmonella gallinarum - 1 ml. of an 18 hour broth culture of S.gallinarum (low virulence strain 6B, Assoku, 1969) was injected intramuscularly.

Chickens were bled by wing vein puncture at the intervals indicated in the results section, and the serum separated and stored at -20°C until antibody determinations were made.

Antibody Determinations

Serum antibody titres to BSA were determined as antigen-binding capacity by the Farr technique (Farr, 1958). Briefly, antisera were diluted 1/10 in borate buffer and any further dilutions made in 1/10 normal chicken serum/borate. For the test 0.5 ml. of diluted antiserum was

mixed with 0.5 ml. ^{131}I BSA (0.2% protein N). After 24 hours 1 ml. 100% Ammonium sulphate was added to give a 50% saturated solution, and the mixture stirred and centrifuged for 30 minutes at 3000 g. The supernatant was discarded and a further 3 ml. 50% saturated ammonium sulphate added. After stirring and centrifugation for another 30 minutes, the supernatant was again poured off and the ^{131}I activity counted in the deposit. For a positive control, dilutions of a standard anti-BSA antiserum were tested, and for a negative control to give the background "entrapment" count, 20% trichloroacetic acid precipitated normal serum was used. Titres were expressed as per cent antigen bound by 0.5 ml. of a 1/10 dilution of test serum using 0.2 % N per test.

Antibody titres to sheep red blood cells were measured by direct haemagglutination and antiglobulin haemagglutination using anti-whole chicken serum and specific anti- γ and anti- μ antiglobulin reagents. All antiglobulin reagents were inactivated and twice absorbed with sheep red blood cells before use. The methods for haemagglutination have already been described in Section II.

Anti-S.gallinarum antibody was measured by a haemagglutination test using S.gallinarum polysaccharide sensitised fowl red cells. This test has also been previously described.

RESULTS

Antibody titres to BSA for normal and bursectomised birds are shown in Fig. 38. The bursectomised birds were divided into three groups according to immunoglobulin status. Chickens were given 2 intravenous doses of antigen with a 4 week interval between them. Blood samples were taken on the 3rd, 6th, 9th, 16th and 24th day after each injection. All control birds showed substantial titres in both the primary and secondary response, the peak for the secondary response being almost twice that of the primary. Only 3 out of 13 bursectomised birds responded. Two of these showed only secondary responses which were of much shorter duration and had very much lower titres than in the control group. These 2 birds both had higher than normal serum IgM levels but no IgG before the antigen injections were given. One bird, which had substantial IgG and IgM serum levels, showed a high antibody titre in the primary response. It died before the second injection and post mortem examination revealed a small residual bursal remnant.

The peak direct and indirect HA titres for sheep red cells are shown in Table 10. The bursectomised birds are again divided into three groups. The schedule of inoculation and blood sampling was the same as for the BSA experiment.

All control birds had substantial direct HA titres during both primary and secondary responses, and very

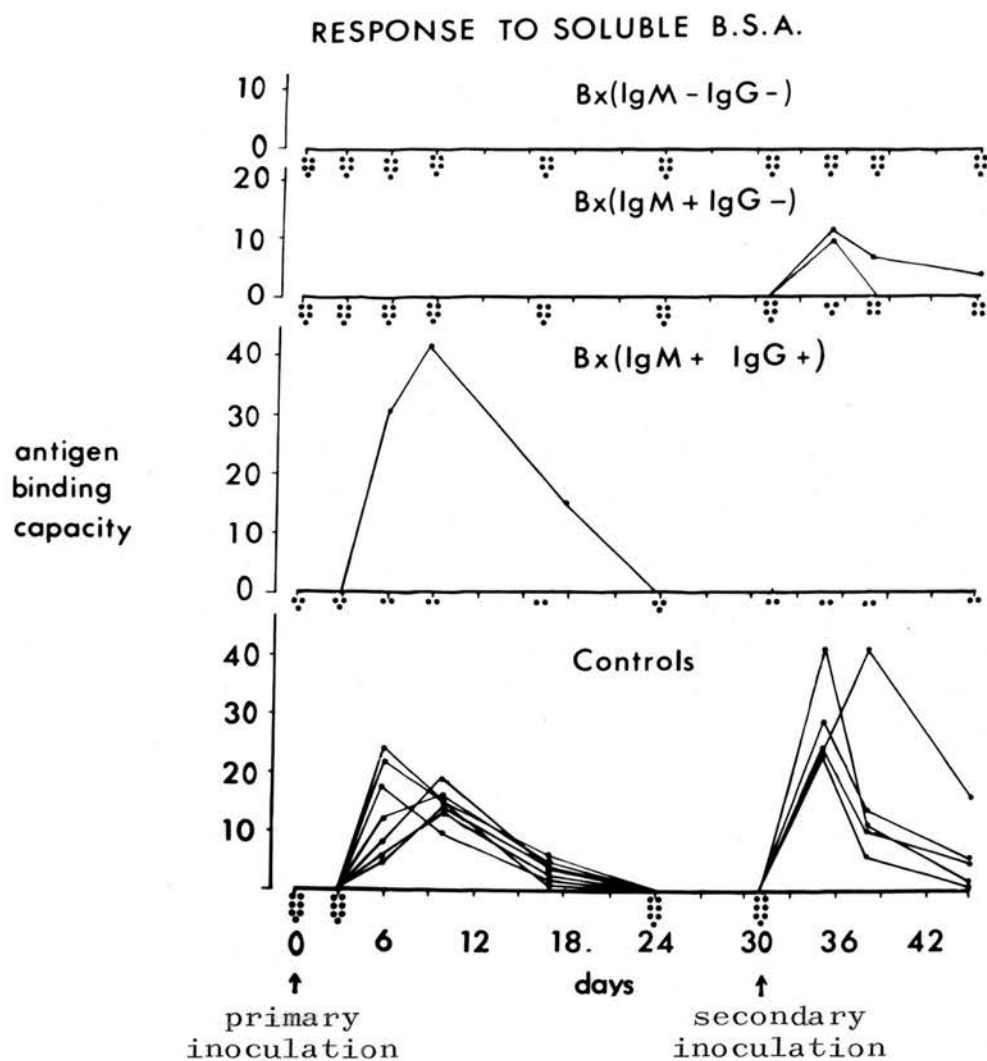


Figure 38.

Antibody titres to BSA in normal and bursectomised birds.

Table 10 : The Response of Normal and Bursectomised Chickens to Sheep Erythrocytes.

| CHICKEN NO. | Ig. STATUS | | PRIMARY RESPONSE | | | SECONDARY RESPONSE | | |
|-------------|------------|-----|-------------------------------|---------|---|-------------------------------|-----------|---|
| | IgG | IgM | Reciprocal direct HA titre | Awhs | Reciprocal antiglobulin titre AIGG AIGM | Reciprocal direct HA titre | Awhs | Reciprocal antiglobulin titre AIGG AIGM |
| 398 normal | + | + | 64 | 4,096 | | dead | | |
| R3664 " | + | + | 256 | 16,000 | | 512 | 32,000 | |
| R3691 " | + | + | 512 | 16,000 | | 512 | 163,000 | |
| R3667 " | + | + | 1024 | 32,768 | | 512 | 1,048,576 | |
| R3689 " | + | + | 320 | 40,960 | | 1,024 | 131,072 | |
| R3699 " | + | + | 512 | 131,072 | | 2,048 | 524,288 | |
| B 185 Bx | - | - | 0 | 4 | 0 | 0 | 4 | 0 |
| B 201 " | - | - | 0 | 2 | 0 | dead | | |
| R3682 " | - | - | 0 | 128 | 16 | " | | |
| A 238 " | - | - | 0 | 0 | | " | | |
| B 182 Bx | - | + | 0 | 0 | | 0 | 0 | |
| B 183 " | - | + | 2 | 8 | 0 | 8 | 128 | 64 |
| A 242 " | - | + | 0 | 8 | 2 | dead | | 128 |
| R3673 Bx | + | + | 0 | 0 | | 0 | 0 | |
| R3674 " | + | + | 0 | 0 | | dead | | |
| R3675 " | + | + | 0 | 0 | | 0 | 0 | |

high antiglobulin HA titres. Five bursectomised birds out of a total of 10 produced antibody during the primary response, all of which were of very low level and could only be detected by the antiglobulin method. Only 2 out of the 5 birds survived long enough to be restimulated and both showed a secondary response - again of a very low order.

The bursectomised birds which did respond to this antigen were distributed in the IgG-ve IgM-ve and IgG-ve IgM+ve groups. It was notable that none belonged to the group which had substantial initial levels of both immunoglobulins.

When the antiglobulin tests were repeated for these responding birds, using specific anti- γ or anti- μ antisera, the HA activity appeared to be due to IgG antibodies in the primary response and to both IgG and IgM in the secondary response. In 4 cases no titre was recorded with either of the specific antiglobulin reagents although low titres had been recorded with anti-whole chicken serum.

This is difficult to explain but might indicate that another antibody class could be involved. However, with such low titres it is quite possible that the test with the specific antiglobulin reagents was not sufficiently sensitive, although both the specificity and strength of the anti- γ and anti- μ antiglobulin sera have been extensively tested as described in Section V.

Of the 7 bursectomised birds infected by intramuscular injection of live S.gallinarum organisms, 4 had produced antibodies when tested 7 weeks after infection (Table 11). Of these 3 showed both direct and indirect HA titres, whilst the 4th had a low titre of IgG antibodies detectable only by the antiglobulin test. In contrast all control birds responded with high direct and indirect IgG and IgM HA titres. Although the direct titres recorded for bursectomised birds were slightly lower than for controls, the indirect titres for these birds were within the normal range.

In this case, in contrast with the sheep red blood cell antigen the responding bursectomised birds were those which had initially produced serum IgM or IgG and IgM. As with BSA none of the totally deficient group produced antibodies. All 4 birds responding to Salmonella produced IgG antibodies, although only 3 had initially had detectable amounts of IgG in their sera. These 3 had in fact synthesised both IgM and IgG at 8 weeks of age, but one produced no detectable direct HA or IgM antibodies. The 4th bird, which belonged to the IgG- IgM+ group produced no IgM antibodies, but had substantial direct HA and indirect IgG titres.

Both control and bursectomised birds showed slight evidence of infection in the first week after injection of the organisms with symptoms of malaise and slight diarrhoea, but all quickly recovered.

Table 11: Antibody responses of normal and bursectomised birds to live S.gallinarum.

| Chicken No. | Ig Status | | Direct HA titre | Antiglobulin HA titre | |
|-------------|-----------|--------|--------------------|--------------------------|----------------|
| | IgG | IgM | | anti- μ | anti- γ |
| A 41 normal | | | 640 | 2,560 | 1,280 |
| A 42 " | | | 5,120 | 20,480 | 40,960 |
| A 43 " | | | 320 | 1,280 | 5,120 |
| A 44 " | | | 640 | 5,120 | 2,560 |
| A 45 " | | | 640 | 5,120 | 5,120 |
| A 46 " | | | 5,120 | 81,920 | 20,480 |
| A 47 " | | | 2,560 | 5,120 | 20,480 |
| 12 Bx | - | - | 0 | 0 | 0 |
| 16 " | - | - + | 0 | 0 | 0 |
| 1 " | - | + | 160 | 0 | 1280 |
| 18 " | - | + | 0 | 0 | 0 |
| 2 " | + | + | 320 | 5,120 | 10,240 |
| 3 " | + | + | 0 | 0 | 320 |
| 5 " | + | + | 320 | 1,280 | 10,240 |

DISCUSSION

Once again, as others have reported (Pierce et al., 1966; Warner et al., 1969; Cain et al., 1969), no direct relationship has been shown to exist between serum immunoglobulin levels in bursectomised birds and antibody responsiveness to several antigens.

Although direct comparisons between the birds infected with Salmonella and those given BSA or sheep red cells may not be valid because of the different techniques used for bursectomy, differences have been observed in the responses to the 3 types of antigen investigated. (Table 12).

| Antigen | Proportion of bursectomised birds responding | | | |
|-----------------------------|--|--------------------|--------------------|--------------------|
| | Total | IgG- IgM- group | IgG- IgM+ group | IgG+ IgM+ group |
| BSA | 3/13 | 0/5 | 2/5 | (1/3) |
| Sheep red blood cells | 5/10 | 3/4 | 2/3 | 0/3 |
| Salmonella gallinarum | 4/7 | 0/2 | 1/2 | 3/3 |

Table 12: The proportion of bursectomised birds responding to different antigens.

Essentially, responsiveness to BSA and sheep red blood cells appears to have been eliminated by bursectomy prior to hatching. However, as would be expected, the few bursectomised birds which did show antibody responses to BSA were those which had previously had measurable serum

IgM or IgG and IgM levels. In the experiment with sheep red cells as antigen, on the other hand, a notable difference was that the responding bursectomised birds were found in the immunoglobulin-deficient groups.

Even bursectomy after hatching resulted in the total elimination of responsiveness to S.gallinarum in a proportion of the chickens tested. However, overall a higher proportion of bursectomised birds produced antibody following this particular treatment, and their titres were near normal levels. As for BSA the bursectomised birds with antibodies against S.gallinarum polysaccharide were those with early detectable serum immunoglobulins.

Whereas most other workers have reported that specific antibody produced by bursectomised birds is mainly IgM, in this experiment, where antibodies have been typed, more birds produced IgG than IgM antibodies, even in some cases where serum IgG had not previously been detectable. Cain et al., (1969) reported a similar finding when sheep red cells were injected into embryonically bursectomised birds. In their experiment, as in ours, some birds severely deficient in serum IgG produced IgG antibodies.

Although the antiglobulin test is a more sensitive method than single radial diffusion, and therefore it is quite probable that antibodies will be detected where serum immunoglobulins appear to be absent, nevertheless it is difficult to explain why, as happened with birds

responding to S.gallinarum, IgG antibody and not IgM antibody was produced by a bird which had had detectable serum IgM but not IgG.

To resolve these anomalies very much larger groups of birds would have to be employed, which were unavailable for the present studies. However, possible reasons can be suggested in the light of current theory. One such suggestion is as follows: IgM-producing cells secrete IgM into the serum without prior antigenic stimulation and multiplication, whereas cells capable of IgG production only begin to multiply and secrete IgG after stimulation. Thus in bursectomised birds where potential IgM or IgG secreting cells are of limited number and specificities, some cells with IgG secreting potential may be present undetected in the peripheral tissues, and if an appropriate antigen is administered, cells present with that specificity multiply rapidly and secrete IgG antibody. Although many more potential IgM cells are present in these birds, and IgM is constantly being secreted into the serum, there may be few specific for the antigen under test, and without a rapid multiplication stage, IgM antibody for that antigen would not be detectable in the serum.

A larger proportion of bursectomised birds responded to *Salmonella* polysaccharide and sheep erythrocytes than to BSA, although the former 2 are so-called bursa-dependent antigens and the latter is

thymus-dependent. This is not surprising, however, since whether or not "T" cells are involved in an antibody response, specific "B" cells must be present for antibody production. Red blood cells and live bacteria are more potent and complex antigens, liable to stimulate a wider range of competent cells than a simple protein such as BSA.

In conclusion, the main findings which emerged from this study were that: (1) the presence or absence of serum immunoglobulins in bursectomised birds did not appear to be related to the ability of the bird to respond to any of the antigens used, and (2) the absence of immunoglobulins in the serum did not necessarily imply that the bird was totally unable to respond to antigen, and (3) apart from the individual anomalies described, bursectomy before hatching appeared to eliminate responsiveness to both antigens tested, whereas neonatal bursectomy had a less certain effect.

SECTION V

STUDIES ON IMMUNOGLOBULIN FUNCTION

IMMUNOGLOBULINS ON THE SURFACE OF LYMPHOCYTES

INTRODUCTION

There has been a great deal of interest in recent years in the "receptor" function of immunoglobulin molecules on the surface of antigen sensitive cells. The "receptor hypothesis" was put forward notably by Mitchison (1967, 1968) and the basic assumption made was that nothing except antibody recognizes antigen. This suggests that all antigen sensitive cells, and therefore both bursa dependent and thymus dependent lymphocytes, recognize and react with their antigen through an antibody type molecule located on their surface.

Although there is now a good deal of evidence, both direct visual and indirect, that immunoglobulin molecules are present on the surface of "B" lymphocytes, increasing doubts have been raised about their presence on "T" lymphocytes. The evidence for immunoglobulin as the "T" cell receptor has been reviewed by Greaves (1970) and is based mainly on indirect methods whereby thymus dependent cellular immune reactions have been blocked by anti-immunoglobulin sera. A more direct method in which the formation of sheep red cell rosettes with "T" lymphocytes was prevented by prior treatment with anti-immunoglobulin serum, relied on identification of "T" cells by the θ antigen.

For the investigation of this problem, the chicken is an experimental animal of unique value, since the two populations of lymphocytes can be studied independently

following the removal of one or other of the central lymphoid organs. Thus "T" lymphocytes can be obtained free from "B" cells and, since serum immunoglobulins have been eliminated, without the possibility of the presence of passively absorbed antibody on their surfaces. The use of anti-lymphocytic sera, anti- θ serum, etc., with the inherent problems of specificity, is unnecessary.

It was, therefore, decided to attempt to demonstrate immunoglobulin determinants on the surface of chicken lymphocytes, and to use bursectomised agammaglobulinaemic and thymectomised chickens to compare the two populations ("B" and "T") with respect to these determinants.

The method of detection chosen was the mixed anti-globulin reaction of Coombs et al., (1970a). By this method immunoglobulin determinants have been demonstrated on the surface of lymphocytes from rabbits (Coombs et al., 1970a), humans (Coombs et al., 1969) and mice (Coombs et al., 1970b). The method has several advantages over others used for the same purpose: (a) non-living lymphocytes are used and therefore lymphocyte suspensions can be prepared in advance and stored either at -70°C or over liquid nitrogen, whereas for both fluorescent antibody staining and transformation studies, freshly prepared viable suspensions are required; (b) normal lymphocytes rather than specifically sensitised cells are required, thus eliminating an antigen inoculation procedure, which is necessary for anti-immunoglobulin inhibition methods.

MATERIALS AND METHODS

Animals - All chickens used for these experiments were over two months of age.

Bursectomy was carried out surgically on the 17th day of incubation as previously described. Only chickens with no IgG or IgM detectable by SRD at 3 months of age were used.

Thymectomy was performed on the day of hatching. The chickens were anaesthetised by halothane inhalation, a longitudinal incision was made down the mid-line of the dorsal aspect of the neck, and the lobules of thymus were removed by suction using a blunt ended pasteur pipette attached to a vacuum pump. A hole was made in the side of the pipette so that the vacuum pressure could be regulated. The incision was closed with nylon sutures which were removed 1 week later. On the day following the operation, the birds were given 650 R whole body X-irradiation. The procedure was as previously described in Section IV for neonatally bursectomised chicks.

Preparation of Lymphocyte Suspensions

Peripheral Lymphocytes

Chickens were bled by cardiac puncture and the blood quickly defibrinated by mechanical stirring in a siliconised bottle with a non-siliconised U-shaped glass rod. By this procedure the thrombocytes are removed along with the fibrin clot. 50 mg carbonyl iron was then added and the sample rotated slowly for 1 hour at 39°C. The blood was then centrifuged in 12 x 0.6 m

tubes at approximately 100 g for 5-8 minutes until the red cells sedimented, and the plasma containing mainly lymphocytes was pipetted into siliconised Wasserman tubes and passed through the jaws of a powerful magnet (Eclipse major) to remove cells which had phagocytosed the carbonyl iron. The lymphocytes were recovered from the supernatant by centrifugation at 1500 rpm and washed three times in phosphate buffered saline (PBS) in 0.8 x 5 or 0.5 x 5 cm siliconised tubes. After each centrifugation the contaminating red cells were left at the bottom of the deposit and the lymphocytes carefully resuspended and transferred to a clean tube. The final preparation, free from red cells, was resuspended in 20% glycerol in PBS and stored at -70°C until testing.

Bursa, spleen and thymus lymphocytes

The organs were not perfused but were washed free of blood and fat in PBS and their capsules removed and discarded. Fine suspensions of cells were then obtained by teasing the tissues in a Petri dish containing PBS and filtering through fine 20μ gauge stainless steel wire mesh filters (Begg, Cousland and Co. Ltd., Glasgow). The cells were then washed and stored in the same way as were the peripheral blood lymphocytes.

Antiglobulin Reagents

Antiserum to chicken IgG was prepared in rabbits by repeated injections of the freeze-dried 0.01 M DEAE cellulose elution fraction from chicken serum, emulsified in Freund's complete adjuvant. The antiserum was

absorbed with freeze-dried serum from agammaglobulinaemic chickens. The absorbed serum had no anti- μ activity but reacted with IgM through light chains.

Anti-Fab antiserum was prepared by absorbing the above anti-IgG antiserum with papain Fc prepared from chicken IgG by the method of Kubo and Benedict (1969). Briefly, IgG was prepared by DEAE cellulose fractionation of 14% Na_2SO_4 precipitated chicken serum globulins. Papain was then added in an enzyme/protein ratio of 1 : 100, along with 0.01 M cysteine and 0.002 M ethylenediaminetetraacetate. After incubation at 37°C for 16 hours recrystallized iodoacetamide was added (0.279 g/ml), and the papain hydrolysate dialysed against frequent changes of distilled water at 4°C for 3 hours. This was followed by a further dialysis for 24 hours against several changes of high salt buffer (1.5 M NaCl, 0.01 M PO_4 , pH 7.0). The precipitate which formed (Fc) was recovered from the supernatant (Fab), washed three times in high salt buffer, and dissolved in distilled water at a concentration of approximately 10 mg/ml. This preparation was dialysed overnight at 4°C against PBS, pH 7.2, and then heated to 37°C. Fc crystals formed rapidly at this temperature. The Fc and Fab preparations were freeze-dried and were examined by double-diffusion with rabbit anti-chicken whole serum (Fig. 39). The Fc preparation contained a single component which was also present in whole IgG and the Fab preparation. A double-diffusion analysis

Figure 39.

Double diffusion analysis of papain Fab and Fc preparations.

Centre well contains rabbit anti-chicken whole serum; outside wells from top clockwise:

1. Whole IgG (0.1 M DEAE cellulose fraction).
2. Fab.
3. Fc.
4. Whole IgG.
5. Fab.
6. Fc.

Figure 40.

Double diffusion analysis of rabbit anti-chicken Fab antiserum.

Centre well contains anti-chicken Fab (prepared by absorption of anti-IgG with agammaglobulinaemic serum and Fc); outside wells from top clockwise:

1. Whole chicken serum.
2. Fab preparation.
3. Fc preparation.
4. Whole chicken serum.
5. Fab.
6. Fc.

Figure 39

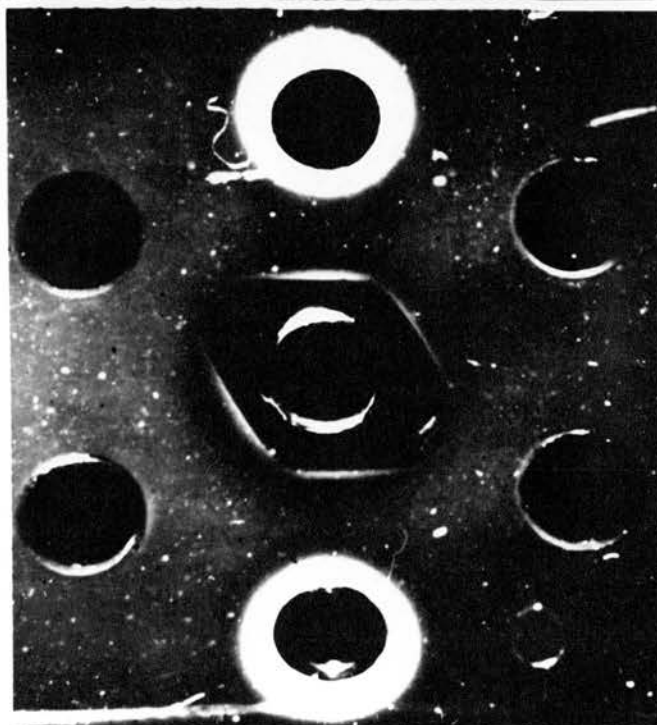
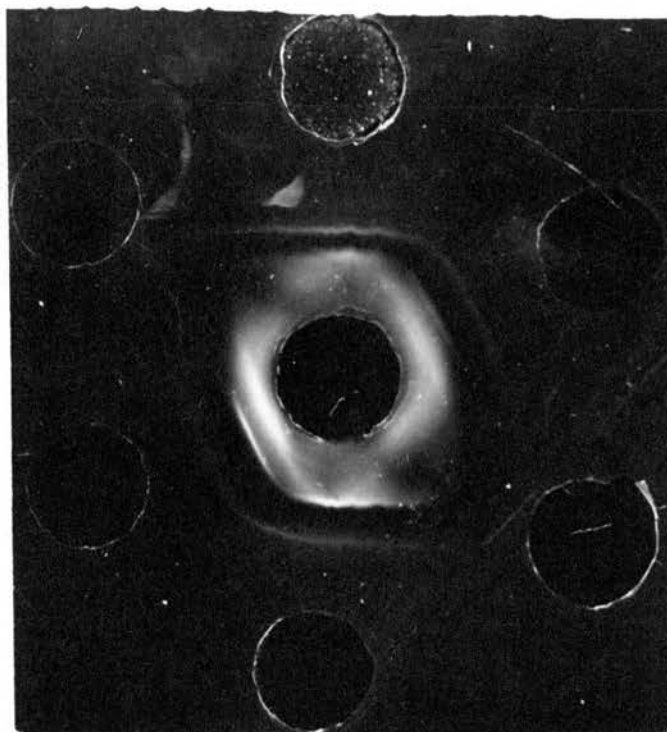


Figure 40

of the anti-IgG antiserum absorbed with agammaglobulinaemic serum and Fc is shown in Fig. 40. The antiserum reacted with IgG and Fab giving a single line, but showed no reaction with Fc.

Specificity for γ chains was obtained by twice absorbing the anti-IgG antiserum with freeze-dried serum from a bursectomised chicken containing high levels of IgM but no IgG.

Antiserum to chicken μ chains was prepared from a polyvalent anti- γ globulin antiserum raised in rabbits by repeated injections of 24% Na_2SO_4 precipitated chicken serum globulins in Freund's complete adjuvant. The antiserum was then absorbed with freeze-dried day old chicken serum which contains IgG but no IgM.

All antiglobulin reagents were heat-inactivated and twice absorbed with well washed sheep and fowl red cells.

Red Cell Indicator System

Chicken anti-sheep red cell serum collected 9 days after a single intravenous injection of 1 ml 10% washed sheep cells, was fractionated on Sephadex G200 and the 1st peak (IgM) antibodies used to sensitise sheep red blood cells for the μ antiglobulin system.

Tertiary chicken anti-sheep red cell serum was collected 10 days after three intravenous injections of 1 ml 10% sheep red cells. This serum was used to sensitise red cells for use in the Fab antiglobulin system.

The Na_2SO_4 precipitated globulins from this tertiary antiserum were fractionated on Sephadex G200 and the second (7S) peak used to sensitise sheep cells for the system.

Sensitisation was achieved by incubating a 2% suspension of sheep red cells in an equal volume of a sub-agglutinating dilution of the various anti-sheep red cell antibody preparations for 1 hour at 4°C . The cells were then washed three times in PBS and resuspended as a 0.4% suspension in PBS containing 0.2% bovine plasma albumin.

Mixed antiglobulin reaction - actual test

The procedure was that described by Coombs et al., (1970a). Essentially, a suspension of lymphocytes recovered from -70°C storage was incubated for 1 hour at 4°C in anti-immunoglobulin reagent, washed 3 times and resuspended in 4 drops of PBS containing 0.2% bovine plasma albumin. They were then centrifuged together with indicator red cells to form a pellet at the bottom of the tube. Half the supernatant was discarded and the deposited cells were resuspended in the remainder and a drop placed, with dye ($\frac{1}{2}\%$ Toluidine blue in methanol), on a siliconised glass slide. The preparation was covered with a siliconised coverslip and the edges sealed with hot paraffin wax.

Lymphocytes with immunoglobulin determinants appeared as rosettes. The appearance of a positive and negative reaction is shown in Figs.41-3. The number of rosetting

Figure 41.

The typical appearance of rosettes in a positive mixed antiglobulin reaction.

x 10 eyepiece.

x 10 objection.

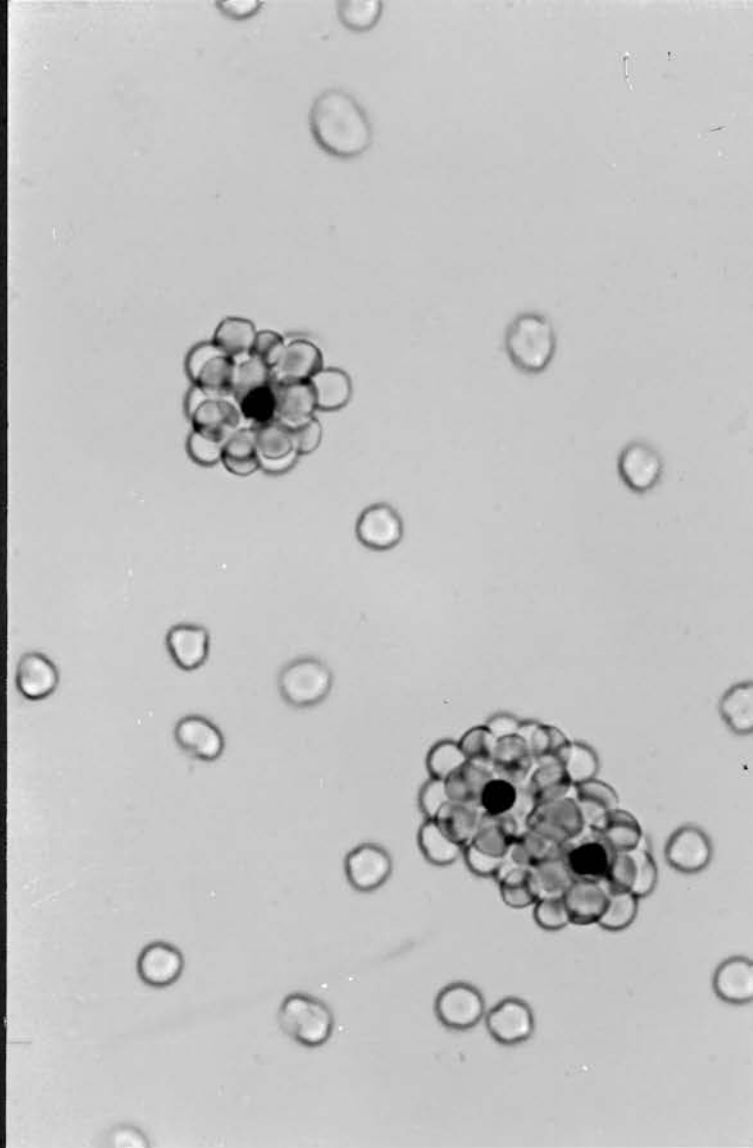
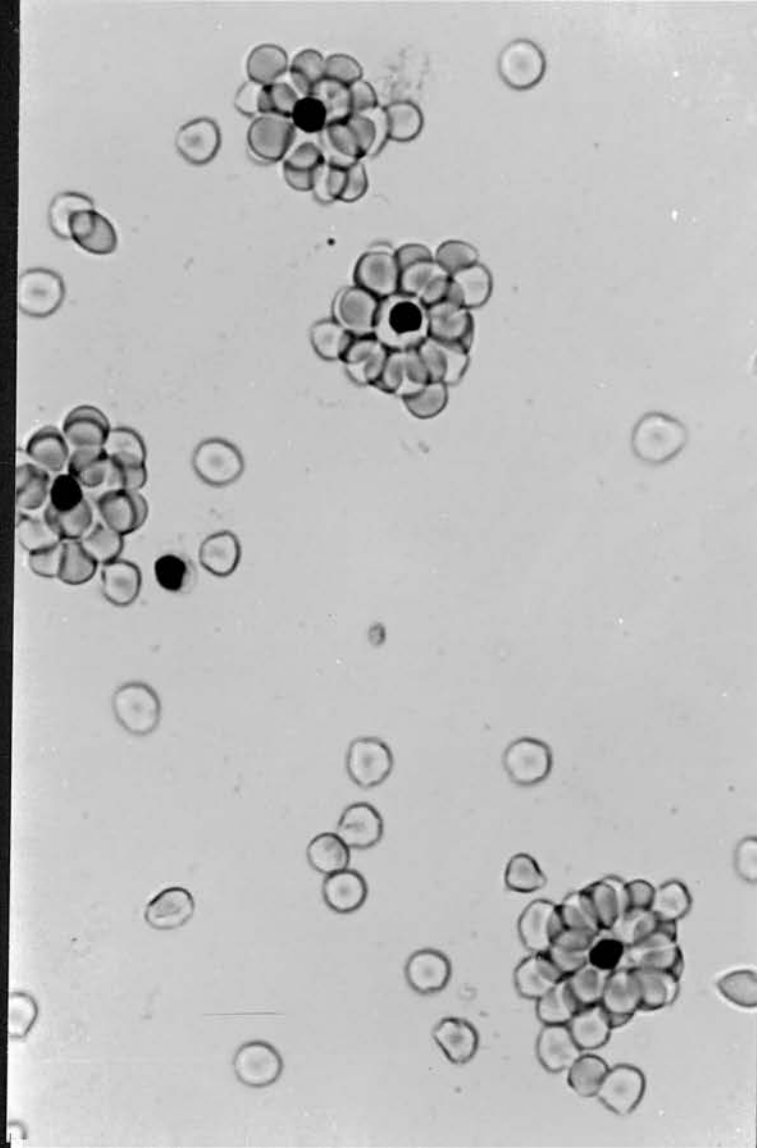


Figure 42.

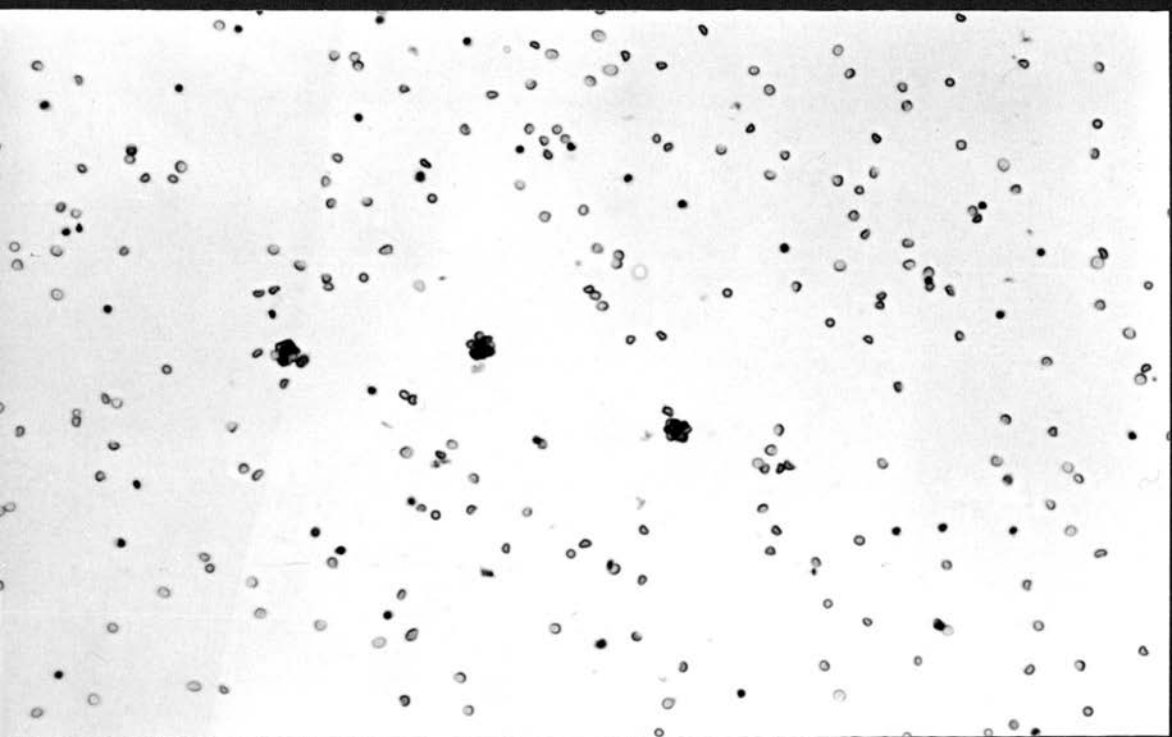
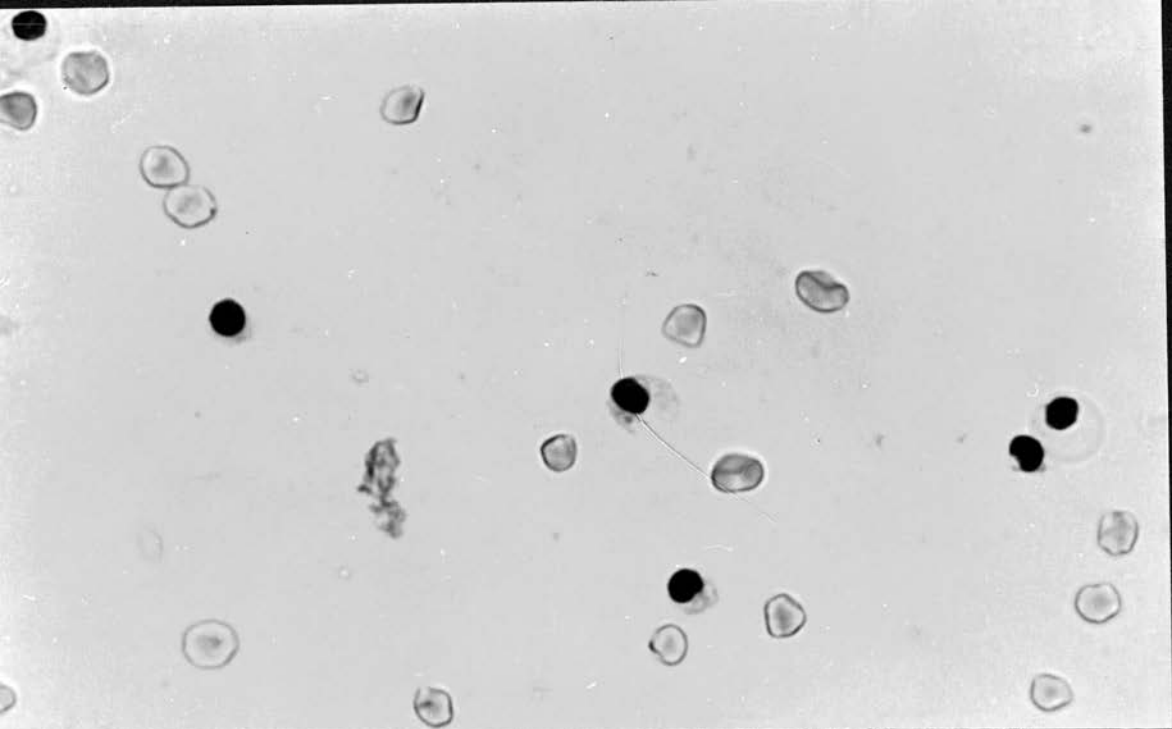
A negative result.

x 10 eyepiece.

x 10 objection.

Figure 43.

**Positive mixed antiglobulin reaction showing
relative numbers of rosetting and non-rosetting
lymphocytes,**



cells was counted as a percentage of the total lymphocytes.

Controls were set up in each test system with lymphocytes incubated in normal rabbit serum instead of anti-immunoglobulin.

EXPERIMENTAL STUDY

Standardization of Reagents

Before the reagents were used in the mixed antiglobulin reaction on lymphocytes, their titres were determined by straight-forward antiglobulin tests on sensitised sheep red cells.

Table 13 shows the agglutinating and sensitising titres of the tertiary anti-sheep red cell serum, of the IgG fraction of this serum and of an IgM fraction of an early antiserum. The sensitisation was detected by the various antiglobulin reagents. The results indicate the specificity of the various systems.

The titres (and specificity) of the antiglobulin reagents were then measured against sheep red cells sensitised with a 1/1000 dilution of the IgG fraction, the IgM fraction and the unfractionated tertiary chicken antiserum to sheep red cells. The results are given in Table 14 and show the class specificity of the anti- γ and anti- μ reagents.

Table 13: The agglutinating and sensitizing titres of tertiary anti-sheep red cell serum, the IgG fraction of the serum and of an IgM fraction of an early antiserum using different antiglobulin reagents.

| Chicken anti red cell sera or fractions | Titre of sheep red cell agglutination with antiglobulin reagent: | | | | |
|---|--|-------------------------|--------------|----------------|-------------|
| | Normal rabbit serum (control) | Anti- whole serum | Anti- Fab | Anti- γ | Anti- μ |
| Tertiary anti-sheep red cell serum (whole serum) | 64 | 8000 | 16000 | 16000 | 64 |
| IgG fraction from tertiary antiserum | 8 | 8000 | 8000 | 16000 | 8 |
| IgM fraction from early antiserum | 320 | 2560 | 16000 | 320 | 2560 |

Table 14: Titration of antiglobulin reagents against sheep red cells sensitised with a 1/1000 dilution of chicken anti-sheep red cell serum or of an IgG or IgM fraction.

| Sheep red cells sensitised with | Titres of antiglobulin reagents | | | |
|---------------------------------|---------------------------------|----------|----------------|-------------|
| | Anti-IgG | Anti-Fab | Anti- γ | Anti- μ |
| Whole antiserum | 5120 | 1280 | 2560 | 0 |
| IgG fraction | 5120 | 640 | 2560 | 0 |
| IgM fraction | 0 | 0 | 0 | 640 |

0 = 10 i.e. starting dilutions were 1/10

Immunoglobulin determinants on the peripheral lymphocytes of normal chickens

Preliminary mixed antiglobulin tests indicated that immunoglobulin determinants could be shown in a number of peripheral lymphocytes. Each reagent was then titrated in tests on lymphocytes to be sure it was being used at a concentration to give an optimal reading (Table 15). Previous reports had shown there to be a marked dose-dependent effect in this reaction (Coombs *et al.*, 1970a). A 1:20 dilution was chosen for the anti-IgG and anti- γ in future tests, the anti-Fab was subsequently used at 1/10, and the anti- μ at 1/7.

Table 16 records the results of tests on the lymphocytes of six normal adult chickens. It was found that between 2.5 and 15.8% of circulating lymphocytes

Table 15: Titration of antiglobulin reagents by the mixed antiglobulin test on lymphocytes from a normal chicken.

| Titre of antiglobulin used to sensitise lymphocytes | % rosetting lymphocytes in undernoted systems | | | |
|--|---|-----|----------|-------|
| | IgG | Fab | γ | μ |
| 1/10 | 11.5 | 10 | 0.5 | 2 |
| 1/20 | 9 | 9.5 | 1 | 1.4 |
| 1/40 | 10 | 2.8 | 1.3 | 1 |
| 1/80 | 7.5 | 5 | 1.1 | 0.5 |

from normal chickens formed rosettes with sensitised sheep cells after incubation in anti-Fab serum (mean approx. 10%). Very similar percentages were recorded in tests using unabsorbed IgG serum as were found with anti-Fab, and so only anti-Fab was used in later experiments. Fewer heavy chain bearing lymphocytes were found - only 0.3 - 2.2% for γ and 1.1 - 11% for μ determinants.

Table 16: Immunoglobulin determinants on the peripheral lymphocytes of normal chickens.

| Chicken number | Per cent reacting lymphocytes with different antiglobulin reagents | | | |
|----------------|--|------|----------|-------|
| | IgG | Fab | γ | μ |
| R3699 | NT | 5.3 | 2.2 | 7.2 |
| 300 | NT | 15.8 | 0.9 | 11 |
| 6892 | 5.5 | 5.7 | 0.5 | 3.4 |
| 396 | 12 | 13.5 | 1.1 | 5.5 |
| R3669 | 5.4 | 2.5 | 0.3 | 3.4 |
| B 160 | 13.5 | 15 | 1.0 | 1.1 |
| Mean | 9.1 | 9.6 | 1 | 5.3 |

NT = not tested

The rosettes were quite unmistakable in appearance as shown in Fig. 41. Only lymphocytes with fully formed rosettes were included in the count as control preparations

incubated in normal rabbit serum occasionally showed lymphocytes with one or two attached erythrocytes. Clumps of red cells where a stained central lymphocyte was not visible were not counted. Normal rabbit serum controls were set up with each lymphocyte preparation but typical rosettes were never seen. More than 1,000 cells were counted on each occasion.

In most cases the percentage of Fab-bearing lymphocytes was greater than the combined percentages of γ and μ . This suggested that another immunoglobulin class might be involved or else on some lymphocytes only the Fab part of the molecule is accessible to the anti-globulin reagent.

Immunoglobulin determinants on peripheral lymphocytes from bursectomised agammaglobulinaemic birds

Table 17 shows the results obtained in parallel tests on the peripheral lymphocytes from 6 agammaglobulinaemic chickens. All 6 birds showed a complete absence of rosettes with anti-Fab, anti- γ and anti- μ (except for one bird - B185 which surprisingly gave 0.2% γ rosettes). The typical appearance of a mixed antiglobulin test with lymphocytes from bursectomised birds is shown in Fig. 43

These findings provide good evidence that the lymphocytes from bursectomised chickens, presumably all thymus derived, do not carry surface immunoglobulin determinants.

Table 17: Immunoglobulin determinants on the peripheral lymphocytes of bursectomised agammaglobulin-aemic chickens.

| Chicken number | Per cent reacting lymphocytes with different antiglobulin reagents. | | | |
|----------------|---|-----|----------|-------|
| | IgG | Fab | γ | μ |
| A 238 | NT | 0 | 0 | 0 |
| B 176 | 0 | 0 | 0 | 0 |
| R3682 | 0 | 0 | 0 | 0 |
| B 192 | 0 | 0 | 0 | 0 |
| B 185 | 0 | 0 | 0.2 | 0 |
| B 201 | NT | 0 | 0 | 0 |

Nevertheless, the possibility has been considered that such determinants are present but may be too few in number, or too deeply buried, to be demonstrated by this technique. In order to investigate this possibility a "piggy back" experiment incorporating a further antiglobulin stage was carried out. For this purpose lymphocyte suspensions from one normal and one bursectomised chicken were used and after carrying out the first treatment as before (either anti Fab in the actual test or normal rabbit serum for its control) the cells were treated with sheep anti rabbit IgG. After incubation and washing the cells were again treated with rabbit anti chicken Fab or normal rabbit serum. Thus a three-layered antiglobulin structure was built up on the lymphocyte surface - a procedure which has been shown by Sell et al., (1970) to enhance lymphocyte transformation. The same technique

was also carried out using the δ and μ systems. The results obtained are shown in Table 18.

Table 18: % rosetting lymphocytes detected by straightforward mixed antiglobulin technique compared with "piggy back" technique.

| | Normal Chicken No. 6892 | | | | Bx Chicken No. 3682 | |
|------------------------------------|-------------------------|----------|-------|-----|---------------------|-----|
| | Fab | δ | μ | NRS | Fab | NRS |
| % rosettes in straightforward test | 5.7 | 0.5 | 3.4 | 0 | 0 | 0 |
| % rosettes in "piggy back" test | 13.6 | 4 | 2 | 0.5 | 1.5 | 1 |

Although the sensitivity of detection of rosettes appeared to be enhanced by the technique, particularly with the Fab system, there was also some loss of specificity as the controls showed a low percentage of rosettes. Despite the increase in sensitivity specifically rosetting lymphocytes could still not be demonstrated in the bursectomised bird as the small number of rosettes observed in the actual test was not significantly higher than that seen in the control system.

Determinants on lymphocytes from thymectomised chickens

In order to investigate the percentage of determinant-bearing lymphocytes in a purely bursa dependent system, peripheral lymphocytes from 5 thymectomised-irradiated

birds were tested with anti-Fab and anti- γ reagents. The results are recorded in Table 19. The mean percentage of cells bearing Fab determinants was approximately 26% - almost three times the mean for the peripheral lymphocytes of normal chickens. Only 2 of the 5 birds had percentages which fell within the normal range, whilst the highest value recorded was 43%. Most of the birds, including those with high percentages of lymphocytes with Fab-determinants had very few or no cells with detectable γ chain determinants.

Table 19: Immunoglobulin determinants on the peripheral lymphocytes of thymectomised chickens.

| Chicken number | Per cent reacting lymphocytes with different antiglobulin reagents. | |
|----------------|---|----------|
| | Fab | γ |
| D100 | 23.0 | 3.0 |
| D 97 | 15.5 | 0 |
| D 98 | 8 | 0 |
| D 99 | 43 | 0 |
| D 96 | 39.2 | 0.5 |
| Mean | 25.7 | 0.7 |

Complete thymectomy is difficult to perform in chickens since the thymus consists of a chain of lobules running down the whole length of each side of the neck in close proximity to the jugular veins. The operation

must be carried out after hatching, thus necessitating whole body irradiation, which makes the procedure less specific. Post mortem examination of two of the thymectomised birds which died revealed remnants of thymic tissue in one (D99) and tumour tissue classified as the reticulum cell sarcoma type in the neck region of the other (D98). The peripheral lymphocyte population in the thymectomised birds is not, therefore, likely to be composed wholly of bursa-dependent cells. The results are in general agreement with the percentage of immunoglobulin determinant bearing lymphocytes detected in lymph nodes and spleens from thymectomised mice by Raff (1970) using indirect fluorescent antibody staining.

Determinants on spleen, thymus and bursa lymphocytes

The results of preliminary tests on bursal lymphocytes and spleen and thymus lymphocytes from normal and bursectomised birds are recorded in Table 20. In the normal birds the percentages of Fab-reacting lymphocytes in the spleen were similar to those found in the circulation, whilst the results for bursal cells were lower, ranging from 3.1 to 9%. These bursal cells were from adult three month old chickens. Between 1.25% and 5.1% of thymus cells formed rosettes in the Fab system. This is a higher proportion than has been reported for rabbits and mice. It is possible that some of these reacting cells may have been derived from peripheral blood within the thymus as neither the

Table 20 : Immunoglobulin determinants on lymphocytes from spleen, bursa and thymus*.

| Chicken number | Treatment | Per cent reacting lymphocytes | | | | | | | | |
|----------------|-----------|-------------------------------|---|-----|-------|-----|-----|--------|----|----|
| | | Thymus | | | Bursa | | | Spleen | | |
| | | Fab | γ | μ | Fab | γ | μ | Fab | γ | μ |
| 99 | None | 1.25 | 0 | 0 | 9 | 0 | 0 | 10 | NT | NT |
| 98 | None | | | | | | | 11.9 | NT | NT |
| 95 | None | 5.1 | 0 | 1.6 | 3.1 | 0 | 1 | | | |
| 100 | None | 3.4 | 0 | 1.5 | 5 | 1.3 | 2.5 | | | |
| 39 | Bx* | 0 | 0 | 0 | | | | | | |
| 172 | Bx | | | | | | | 0 | 0 | 0 |
| 28 | Bx | 0 | 0 | 0 | | | | | | |
| 26 | Bx | | | | | | | 0 | 0 | 0 |

Bx = bursctomised

* the organs were not perfused free of blood

NT = not tested

intact birds nor the organs were perfused before teasing the organs. Surface immunoglobulin was not demonstrable on spleen or thymus lymphocytes from bursectomised chickens.

DISCUSSION

The implication from these results is that thymus dependent lymphocytes in the chicken do not carry surface immunoglobulin determinants, at least not in detectable amounts.

Although at the time this work was undertaken, opinion in general favoured the opposite alternative, i.e. that "T" cell antigen receptors were immunoglobulin in nature, there were already reports in the literature which suggested that they might not be, although direct evidence was scarce. Thus Raff, (1970) had found that cells bearing immunoglobulins detectable by immunofluorescence were not θ positive, and that the distribution of θ bearing and immunoglobulin bearing lymphocytes were inversely related, suggesting that the cells constituted two distinct populations. Also in agreement with this were the reports by Alm and Peterson, (1969) and Ivanyi et al., (1969) that anti-immunoglobulin induced transformation of lymphocytes was greatly reduced in bursectomised chicks. Similarly, Ivanyi et al., (1970) showed that chicken thymus cells did not transform under the influence of antiglobulin serum. Earlier in vivo experiments by Silverstein et al., (1963), in which agammaglobulinaemic foetal lambs were injected with anti IgG and subsequently showed normal skin graft rejection, had also indicated that the cells involved in the rejection process ("T" cells) did not rely on immunoglobulin molecules

for recognition of antigens, whilst Schlossman and Levine, (1967) from observations on specificities of antibody and delayed hypersensitivity reactions had concluded "that a preformed cell-fixed or circulating antibody is not the mediator of the delayed response". Very recently many more reports have been published which are in agreement with this finding that "T" lymphocytes do not bear surface immunoglobulins.

Rabellino and Grey, (1971) and Kincade et al., (1971), using a fluorescent antibody staining technique, and Bankhurst and Warner, (1972), using autoradiography, have also failed to detect immunoglobulins on the surface of lymphocytes from bursectomised chickens. The percentages of determinant bearing lymphocytes in normal chickens recorded by these authors are higher than have been recorded here by the mixed antiglobulin reaction. This may be because the latter method of detection of immunoglobulin determinants selectively picks up only those determinants on the lymphocyte surface which are identical with the ones on the sensitised indicator red cells. The fluorescent antibody method could detect a variety of determinants on the surface immunoglobulin molecules and would also allow cells with only a few determinants confined to one area of the membrane to be counted.

Another method of detecting surface immunoglobulins, "reverse immune cytoadherence" reported by Paraskevas et al., (1971), also failed to pick up immunoglobulins

on mouse or human thymus cells although rosette-forming cells were demonstrated in the blood, bone marrow, lymph nodes and spleens. Autoradiography studies on mouse lymphocytes by Jones et al., (1971) gave similar results.

All the direct evidence, therefore, which has included many different methods for the demonstration of cell surface immunoglobulins, suggests that "T" lymphocytes do not carry these determinants.

However, the possibility still exists that immunoglobulin molecules are present on these cells but that they are either too few or too inaccessible in position to be demonstrable by the direct techniques available.

Convincing evidence for the presence of immunoglobulins on "T" cells is scarce, but some results have been published which are difficult to refute.

As already mentioned the main evidence put forward by Greaves, (1970) was based on sheep red cell rosette inhibition studies and the blocking of tuberculin-induced cell transformation by anti-immunoglobulins. Some data will be presented below which suggests that rosette-forming cells are exclusively bursa dependent, thus tending to invalidate the first line of evidence. However, the inhibition of tuberculin-induced transformation, and recent experiments by Basten et al., (1971) where "T" cell "suicide" by specific reaction of these cells with radioactive antigen was blocked by anti light chain

antiserum remain at present unchallenged. Experiments showing inhibition of transfer of delayed hypersensitivity by pretreatment of sensitised cells with anti light chain serum (Mason and Warner, 1970) provide further evidence, although a possible explanation of these results is that the cells being inhibited were cytophilic antibody coated macrophages rather than "T" lymphocytes since 70% inhibition of peritoneal exudate cells was achieved whereas the inhibition of lymph node cells was "only marginal".

Autoradiography studies on foetal thymus cells by Dwyer and Mackay, (1970) and Dwyer and Warner, (1971) indicated that many of these cells bound antigen, and that the binding was inhibited by anti-immunoglobulin serum (Dwyer and Warner, 1971). However, many more antigen binding cells were present in foetal bursa suspensions and antigen binding occurred earlier in the bursa than elsewhere, so that the antigen binding cells in the thymus could possibly have originated from the bursa.

The complicating factor in all these experiments is the fact that circulating antibody (and therefore the possibility of cytophilic antibody) is present in all the systems and that it is difficult in any mammalian system to be completely certain of the purity of the cell suspensions ("B" and "T") being studied.

It is obvious, therefore, that the bursectomised agammaglobulinaemic chicken is the ideal experimental animal in which to study the antigen receptors of the thymus dependent lymphocyte.

STUDIES ON "T" LYMPHOCYTE RECEPTORS

Much of the evidence quoted above suggests that the antigen-receptor of the thymus dependent system is not immunoglobulin in nature, and so several groups have embarked on experiments to discover what type of molecule is involved. Affinity labelling methods have been used by Hill, (1971) to trace the reactive molecules on lymphoid cells from guinea-pigs sensitised to give delayed hypersensitivity reactions. Two specifically labelled peaks, one of the MW 39,000 - 45,000 and the other of MW 22,000 - 25,000 were isolated by gel filtration methods from solubilised cell membranes, and components from both peaks were capable of passively sensitising normal guinea-pigs (Hill and Nissen, 1971).

Non-immunoglobulin factors released into the plasma by lethal irradiation of sensitised guinea-pigs have also been shown to passively transfer delayed hypersensitivity reactions in guinea-pigs by Dupuy and Good, (1970). These factors are specific but are not γ 1 or γ 2 antibodies (Dupuy and Good, 1971), and the authors suggest that they may be the "thymus-dependent antibody" (Perey et al., 1970a).

However, whilst experiments of the above nature are of great value, it is nevertheless vital at this time to establish without doubt that classical immunoglobulins are not involved in "T" cell antigen-recognition. For this purpose it was decided to study cellular immune reactions of lymphocytes in agammaglobulinaemic bursectomised birds,

and to try to inhibit these with anti-immunoglobulin sera. The work is still continuing, but initial results can be reported here.

The most simple approach was to attempt to inhibit antigen binding of lymphocytes, known without doubt to be thymus-dependent. The system chosen was sheep red cell immunocytoadherence which Greaves et al., (1970) have reported involves both "T" and "B" lymphocytes. According to these authors the maximum proportion of rosetting "T" lymphocytes (detected by θ antigen) is obtained in non-immunised mice, 3 months after sheep red cell inoculation, or when sheep cell inoculation is followed a few days later by hyperimmune serum. None of these alternatives were useful for this work since (a) chickens were found to have very low or negative background rosette counts for sheep red cells (from early experiments), (b) the injection of hyperimmune serum would abolish the benefits of agammaglobulinaemia and allow the possible complication of cytophilic antibody, and (c) for a pilot experiment an interval of 3 months between injection and testing would be impractical. It was thus decided to look for rosetting lymphocytes in bursectomised birds at various times after injection of sheep cells by different routes. The method used for the test was as follows. Lymphocyte suspensions were prepared as previously described, the cells washed 3 times in PBS, counted and adjusted to a suspension of approximately 4 million / 1 ml. 0.02 ml.

volumes were then incubated for 1 hour at 4°C with an equal volume of normal rabbit serum or polyvalent rabbit anti-chicken immunoglobulin serum both of which had previously been absorbed with sheep cells. 0.02 ml. of 0.5% suspension of sheep red cells was then added and the mixture centrifuged for 2 minutes at 100 g. Half the supernatant was removed, the cells resuspended in the remainder and the whole volume placed on a siliconed slide with a waxed siliconed coverslip. The total number of rosettes in the preparation was counted. Their appearance is shown in Fig. 44.

Lymphocyte preparations from normal and bursectomised chickens were tested at various intervals after one or two intravenous injections of sheep cells or one intramuscular injection of 10% red cells in Freund's complete adjuvant. The results are shown in table 21.

From these results it is apparent that with the inoculation schedules used, the great bulk, if not all of the rosetting lymphocytes, were bursa-derived. The very small number of rosettes seen in the bursectomised agammaglobulinaemic birds 5 days after intramuscular injection of sheep red cells in Freund's complete adjuvant were not inhibitable with anti-immunoglobulin serum and might possibly have involved thymus-dependent cells. However, the numbers were so small that antigen-binding inhibition studies of this nature do not appear to be practicable. Other authors have recently also raised doubts as to whether "T" lymphocytes can form rosettes

Figure 44a.

A negative preparation.

Figure 44b.

Rosette-forming cells in the circulation of normal chickens after sheep red cell inoculation.

x 10 eyepiece.

x 10 objection.

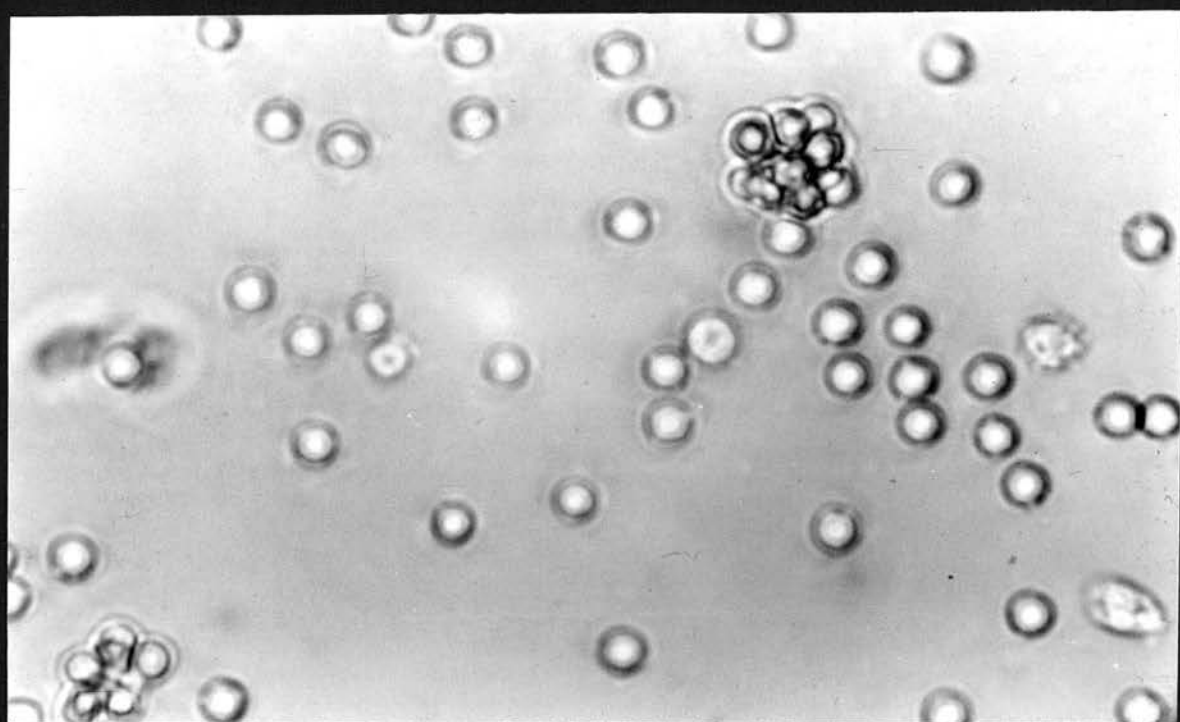
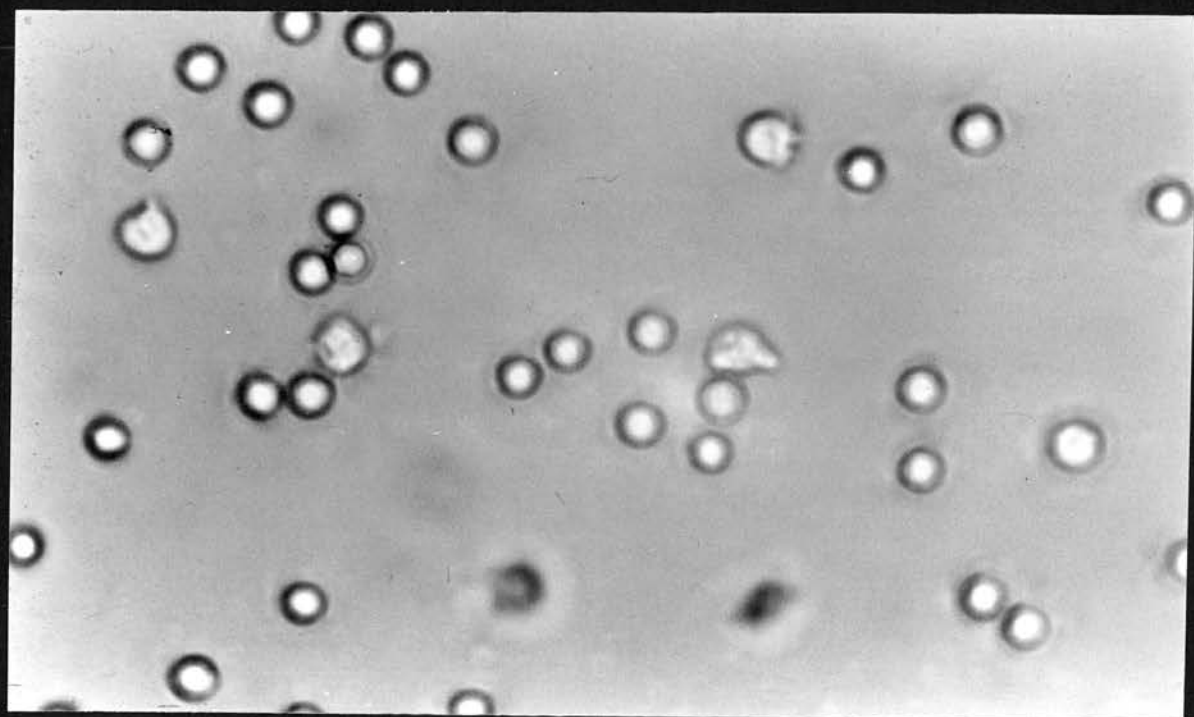


Table 21: Rosette-forming cells in the circulation of normal and Bx birds following various schedules of sheep red cell inoculations.

| Inoculation details | Chicken | Ig Status IgG IgM | Days post injection | No. rosettes per 1000 lymphocytes (NRS treatment) | No. rosettes (A-Ig) | % Inhibition |
|--|-------------|----------------------|---------------------|---|---------------------|--------------|
| 1 intravenous injection of 1 ml 10% sheep red cells | C1 control | ++ | 10 | 15 | 0.15 | 99 |
| | 12 Bx | - | 10 | 0.00006 | | |
| | 166 Bx | - | 45 | 0.01 | | |
| | 17 Bx | - | 10 | 0.00005 | | |
| | 33 Bx | + | 10 | 0.00001 | | |
| | 144 Bx | ++ | 76 | 0 | | |
| 2 intravenous injections of 1 ml 10% sheep red cells | 184 control | ++ | 10 | 16 | | |
| | 117 Bx | - | 13 | 0 | | |
| | 147 Bx | - | 24 | 0.5 | | |
| | 164 Bx | ++ | 10 | 0.05 | | |
| | 171 Bx | ++ | 10 | 20 | | |
| | 121 Bx | ++ | 6 | 22 | | |

Continued overleaf

Table 21 Continued

| Inoculation details | Chicken | Ig Status IgG IgM | Days post injection | No. rosettes per 1000 lymphocytes (NRS treatment) | No. rosettes (A-Ig) | % Inhibition |
|---|------------|----------------------|------------------------|---|------------------------|-----------------|
| 1 intramuscular injection of 1 ml 10% sheep red cells in Freund's complete adjuvant | C2 control | ++ | 3 | 0.02 | 0 | 100 |
| | C3 control | ++ | 3 | 0.15 | 0 | 100 |
| | 12 Bx | - | 3 | 0 | | |
| | 26 Bx | - | 3 | 0 | | |
| | 33 Bx | + | 3 | 0 | | |
| | 37 Bx | +- | 3 | 0.01 | 0 | 100 |
| | C2 | - | 5 | 0.04 | 0 | 100 |
| | C3 | | 5 | 0.13 | 0.02 | 85 |
| | 12 | | 5 | 0 | 0 | |
| | 26 | | 5 | 0.02 | 0.04 | 0 |
| | 33 | | 5 | 0 | 0.02 | 0 |
| | 37 | | 5 | 0.05 | 0.12 | 0 |
| | C2 | | 7 | 0.25 | 0 | 100 |
| | C3 | | 7 | 0.49 | 0.03 | 94 |
| | 12 | | 7 | 0.02 | 0 | 100 |
| | 26 | | 7 | 0 | 0 | |
| | 33 | | 7 | 0.01 | 0 | 100 |
| | 37 | | 7 | 0 | 0 | |

with sheep red cells. Roberts et al., (1971) immunised guinea-pigs with DNP-keyhole limpet haemocyanin (DNP-KLH) in Freund's complete adjuvant. Cells which formed rosettes with red cells coated with DNP conjugates, and antibody to DNP-KLH were detected, and delayed hypersensitivity reactions appeared to DNP-KLH, and KLH alone. However no cells formed rosettes with red cells coated with KLH alone, and no antibody was formed to this antigen, although a good delayed hypersensitivity response had been elicited, suggesting that the thymus-dependent lymphocytes involved in delayed hypersensitivity reactions do not form rosettes. Similar results have been found in a tuberculin system by Coombs (unpublished results) and are being confirmed in this laboratory with agammaglobulinaemic and normal chickens.

SECTION VI

GENERAL DISCUSSION

The work presented in this thesis has been discussed in detail in the individual sections. However, several facts and conclusions emerge which bear repetition here, and some interesting aspects worthy of further detailed research suggest themselves.

From the studies on chicken immunoglobulins, although some difficulties arise in the preparation of purified immunoglobulins and especially of specific anti-immunoglobulin sera, it seems to be quite legitimate, and make for much greater simplicity, to refer to these immunoglobulins as IgG and IgM, like their mammalian counterparts. The physico-chemical differences which do exist between chicken and avian antibodies do not seem important enough to merit a new terminology.

Although a third immunoglobulin class has not been isolated and characterised in this work, a third protein has been described which migrates electrophoretically as a γ 1 globulin, appears in the 7S peak in Sephadex G-200 gel filtration and elutes from DEAE cellulose almost exclusively with 0.09 Molar phosphate buffer. This protein does not appear to have antibody properties and its synthesis is not under the control of the bursa of Fabricius.

It would be of great interest to discover whether the synthesis of chicken IgA, which other authors have recently claimed to have isolated, is under the influence of the bursa. If it were not, then the observation

that chickens lacking serum IgG and IgM do not suffer dramatic infections of the gastro-intestinal or respiratory tract, and are not invaded by their intestinal flora, would be easier to comprehend.

A method of surgical bursectomy in ovo has been described which has not hitherto been well documented or widely used. When performed on the 17th day of embryonic development, this method can inhibit completely both IgG and IgM synthesis in up to 30% of operated birds. It has the advantage over all the other methods so far described (including recently described methods involving injection of anti-IgM antiserum into embryos followed by neonatal surgical bursectomy (Kincade et al., 1970), and cyclophosphamide injection into neonatal chickens (Linna et al., 1972)) of being completely specific.

Experiments in which surgical bursectomy was performed on the 17th, 18th or 19th day of incubation and serum immunoglobulin synthesis compared in the 3 groups of birds have established what was suggested earlier by Cooper et al., 1969 and has been shown recently by other methods by Moore and Owen, (1971), that the 17th day of incubation appears to be a critical point in time at which, many cells capable of IgM synthesis having already left the bursa, a switch-over to the development of cells capable of IgG synthesis

occurs within the bursa.

A long-term study of serum immunoglobulin levels in bursectomised birds has shown that even birds which at 8 weeks of age were producing both immunoglobulins, after several months eventually become agammaglobulinaemic. This finding would appear to be of great relevance to theories of cell differentiation, for it suggests either that all immunologically competent precursor cells for antibody production must derive directly from the bursa, or else that only limited multiplication of bursa derived competent cells can occur in the peripheral tissues.

A study of mortality rates in bursectomised chickens has shown that the presence or absence of serum immunoglobulin in bursectomised chickens does not affect their life expectancy, indicating that these immunoglobulins are not necessarily specific antibodies with a protective function. Also, although bursectomy does reduce survival time as compared with normal chickens, the absence of obvious enteric and pulmonary diseases in bursectomised birds suggests that in the fowl other defence mechanisms are as important or more important than circulating antibodies for protection against many infections. The pattern of mortality has been shown to differ in the control and bursectomised groups - 79.3% of all deaths in the normal birds occurring between 7 and 20 weeks of age, compared with only 22.4% in the bursectomised birds.

This period is the most common time for Marek's disease to develop and it is suggested that this disease was the cause of death of a large proportion of the control birds but not of the bursectomised ones. Post mortem histological examination of a random group of birds did not rule out this possibility. Although it has been shown that bursectomised birds can be experimentally infected with Marek's disease virus, and it could be argued that some of the bursectomised group may in fact have died from the disease before 7 weeks of age, this is unlikely since the majority of deaths in bursectomised birds occurred in the first week of life.

Specific antibody responses of bursectomised chickens to BSA, sheep red cells and S.gallinarum have been measured and related to serum immunoglobulin synthesis. Essentially, bursectomy in ovo completely eliminated antibody responses to sheep red cells and BSA in the majority of birds so treated, whilst neonatal bursectomy + irradiation produced a smaller proportion of birds totally unresponsive to S.gallinarum. Some interesting anomalies occurred in these experiments. For instance, for sheep red cells the few responding birds were found in the totally or partially deficient bursectomised groups and not in the group which initially had both IgG and IgM in their sera. Also, the antibody produced against Salmonella was mainly of the IgG type even where

individual birds had previously synthesised only serum IgM. Although possible reasons for these anomalies have been suggested, to resolve the problem fully a very large number of birds would have to be tested. In this connection it would be of value to pursue these observations further and to test the hypothesis of a sequential and stepwise development of specific responsiveness to different antigens by using groups of birds bursectomised surgically at different times before hatching.

The very mild effects of parenteral administration of live S.gallinarum organisms on bursectomised birds emphasises once again the relative unimportance of humoral immunity in certain diseases. An investigation of blood changes in Salmonellosis in these birds which do not produce antibody is expected to confirm the role of antibody in the clinical anaemia seen in this disease (Assoku and Penhale, 1970).

The mixed antiglobulin reaction has been successfully used to demonstrate the presence of immunoglobulin determinants on chicken lymphocytes. A comparison of peripheral lymphocytes from bursectomised agammaglobulin-aemic, normal, and thymectomised birds has revealed that these determinants can only be shown on bursa-dependent lymphocytes. This had not been clearly demonstrated previously, although several groups of workers have since

confirmed these findings using different methods for the detection of immunoglobulins.

Although this suggests that "T" lymphocytes do not carry immunoglobulins, and therefore must have a different type of receptor molecule, it is possible that immunoglobulins are present although not in a position or concentration which allows detection by the methods so far employed.

Although up to 15% of peripheral lymphocytes have been shown to carry Fab determinants, very few heavy chain bearing cells have been found. It would be valuable to extend these studies to include lymphocytes from bursectomised birds with high serum IgM levels but lacking in IgG, to discover whether (a) immunoglobulin bearing cells are present in the circulation in these birds, and (b) if the proportions of Fab γ and μ bearing cells reflect the serum immunoglobulin pattern.

It cannot be too strongly emphasised that the agammaglobulinaemic bursectomised chicken is the ideal system for the study of "T" lymphocytes. Future work in bursectomised chickens on antigen binding to these lymphocytes should provide valuable information on the unresolved problem of the true nature of the "T" lymphocyte receptor.

A major contribution of this work, then, has been to provide the prerequisites for future studies on "T" lymphocytes.

These are:

1. the production of mono-specific anti-immunoglobulin sera;
2. the use of these sera to measure serum immunoglobulin;
3. the development of a method of bursectomy which can provide completely agammaglobulinaemic chickens without affecting any other lymphoid tissues;
4. the development of a method to obtain pure lymphocyte suspensions from chickens and therefore pure "T" lymphocyte suspensions;
5. the provision of good evidence that immunoglobulins are not directly demonstrable on "T" lymphocytes, hence pointing the way towards direct antigen-binding experiments or methods involving the inhibition of cellular immune reactions in agammaglobulinaemic chickens.

REFERENCES

- ACKERMAN, G.A. and KNOUFF, R.A., (1964), in "The Thymus in Immunobiology", ed. by Good, R.A. and Gabrielsen, A.E. Hoeber, N.Y., p.123.
- ALM, G.V. and PETERSON, R.D.A., (1969), J. exp. Med. 129, 1247.
- ARNASON, B.G. and JANKOVIC, B.D., (1967), J. Immun. 99, 917.
- ARNASON, B.G., JANKOVIC, B.D. and WAKSMAN, B.H., (1962), Nature, Lond. 194, 99.
- ASPINALL, R.L. and MEYER, R.K., (1964), in "The Thymus in Immunobiology", ed. by Good, R.A. and Gabrielsen, A.E. Hoeber, N.Y., p.376.
- ASSOKU, R.K., (1969), Ph.D. Thesis, University of Edinburgh.
- ASSOKU, R. and PENHALE, W., (1970), Clin. & Exp. Immunol. 7, 875.
- BAGLIONI, C., (1963), in "Molecular Genetics" (2 pts.), ed. by Taylor, J.H., Academic Press, N.Y., p.405.
- BANG, B.A. and BANG, F.B., (1968), Am. J. Path. 53, 735.
- BANKHURST, A.D., ROUSE, B.T. and WARNER, N.L., (1972), Int. Archs Allergy appl. Immun. 42, 187.
- BANOVITZ, J., SINGER, S.J. and WOLFE, H.R., (1959), J. Immun. 82, 481.
- BANOVITZ, J. and WOLFE, H.R., (1959), J. Immun. 82, 489.
- BASTEN, A., MILLER, J.F.A.P., WARNER, N.L. and PYE, J., (1971), Nature New Biology 231, 104.
- BENEDICT, A.A., (1967), in "Methods in Immunology and

- Immunochemistry" vol. I, ed. by Williams, C. A. and Chase, M.W., Academic Press, N.Y., p.229.
- BENEDICT, A.A., BROWN, R.J. and HERSH, R.T., (1963), J. Immun. 90, 399.
- BIGGS, P.M., (1956), Vet. Rec. 68, 525.
- BIGGS, P.M., (1957), Acta anat. 29, 36.
- BLOCH, K.J., MORSE, H.A. and AUSTEN, K.F., (1968), J. Immun. 101, 650.
- BRANDLY, C.A., MOSES, H.E. and JUNGHER, E.L., (1946), Am. J. vet. Res. 7, 333.
- BULLETIN WORLD HEALTH ORGANISATION, (1964), 30, 447.
- BURMEISTER, B.R., (1955), Proc. Soc. exp. Biol. Med. 88, 153.
- BURNET, F.M., (1969), in "Cellular Immunology" Bk 2, Melbourne University Press, Cambridge, p.374.
- BUXTON, A., (1952), Ph.D. Thesis, University of Cambridge.
- CAIN, W.A., COOPER, M.D. and GOOD, R.A., (1968), Nature, Lond. 217, 87.
- CAIN, W.A., COOPER, M.D., VAN ALTEN, P.J. and GOOD, R.A., (1969), J. Immun. 102, 671.
- CAIN, W.A., WEIDANZ, W.P. and COOPER, M.D., (1967), Fedn Proc. Fedn Am. Socs exp. Biol. 26, 571.
- CHANG, T.S., RHEINS, M.S. and WINTER, A.R., (1957), Poult. Sci. 36, 735.
- CHANG, T.S., RHEINS, M.S. and WINTER, A.R., (1959), Poult. Sci. 38, 174.

- CLAFLIN, A.J., SMITHIES, O. and MEYER, R.K., (1966),
J. Immun., 97, 693.
- CLAMAN, H.N., CHAPERON, E.A., FRASER
(1966), J. Immun., 97, 828.
- COHEN, S. and PORTER, R.R., (1964), Adv. Immunol., 4, 287.
- COOMBS, R.R.A., FEINSTEIN, A. and WILSON, A.B., (1969),
Lancet, II, 1157.
- COOMBS, R.R.A., GURNER, B.W., JANEWAY, C.A., WILSON, B.A.,
GELL, P.G. and KELLUS, A.S., (1970a), Immunology, 18, 417.
- COOMBS, R.R.A., GURNER, B.W., McCONNELL, I. and MUNRO, A.,
(1970b), Int. Archs Allergy appl. Immun., 39, 280.
- COOPER, M.D., CAIN, W.A., VAN ALTEN, P.J. and GOOD, R.A.,
(1969), Int. Archs Allergy appl. Immun., 35, 242.
- COOPER, M.D., GABRIELSEN, A.E., PETERSON, R.D.A. and
GOOD, R.A., (1967), in "Germinal Centres in Immune
Responses", ed. by Cottier, H., Odartchenko, O., Schindler,
R. and Congdon, C.C., Springer-Verlag, Berlin, p.28.
- COOPER, M.D., PEREY, D.Y., GABRIELSEN, A.E., SUTHERLAND,
D.E.R., McKNEALLY, M.F. and GOOD, R.A., (1968), Int.
Archs Allergy appl. Immun., 33, 65.
- COOPER, M.D., PETERSON, R.D.A. and GOOD, R.A., (1965),
Nature, Lond., 205, 143.
- COOPER, M.D., PETERSON, R.D.A., SOUTH, M.A. and GOOD, R.A.,
(1966a), J. exp. Med., 123, 75.
- COOPER, M.D., SCHWARTZ, M.L. and GOOD, R.A., (1966b),
Science, N.Y., 151, 471.
- DENT, P.B. and GOOD, R.A., (1965), Nature, Lond., 207, 491.

- DENT, P.B., PEREY, D.Y.E., COOPER, M.D. and GOOD, R.A., (1968), *J. Immun.* 101, 799.
- DEUTSCH, H.F., NICHOL, J.C. and COHN, M., (1949), *J. Immun.* 63, 195.
- DREESMAN, G.R. and BENEDICT, A.A., (1965a), *J. Immun.* 95, 855.
- DREESMAN, G.R. and BENEDICT, A.A., (1965b), *Proc. natn. Acad. Sci. U.S.A.* 54, 822.
- DREESMAN, G.R., LARSON, C., PINKARD, R.N., GROYN, R.M. and BENEDICT, A.A., (1965), *Proc. Soc. exp. Biol. Med.* 118, 292.
- DUFFUS, W.P.H. and ALLAN, D., (1968), *Immunology*, 15, 653.
- DUFFUS, W.P.H. and ALLAN, D., (1969), *Immunology*, 16, 337.
- DUPUY, J.M. and GOOD, R.A., (1970), *J. Immun.* 105, 1111.
- DUPUY, J.M. and GOOD, R.A., (1971), *J. Immun.* 106, 528.
- DWYER, J.M. and MACKAY, I.R., (1970), *Lancet* I, 1199.
- DWYER, J.M. and WARNER, N.L., (1971), *Nature New Biology*, 229, 210.
- DZIERGOVSKI, S.K., (1901), *Arch. des Sci. Biol.*, St. Petersburg, 8, 211 and 429.
- EDELMEN, G.M. and MARCHALONIS, J.J., (1967), in "Methods in Immunology and Immunochemistry" vol. I, ed. by Williams, C.A. and Chase, M.W., Academic Press, N.Y., p. 405.
- EWING, J. and STRAUSS, I., (1903), *Med. News*, N.Y., 83, 871 and 925.
- FAHEY, J.L. and McKELVEY, E.M., (1965), *J. Immun.* 94, 84.

- FARR, R.S., (1958), *J. infec. Dis.*, 103, 239.
- FERNANDO, W.W.D. and CALNEK, B.W., (1971), *Avian Dis.*, 15, 467.
- FICHTELIUS, K.E., (1960), in *Ciba Foundation Symposium on "Haematopoiesis"*, ed. by Wolstenholme, G.E.W., Churchill, London, p.207.
- FRENCH, V.I., STARK, J.M. and WHITE, R.G., (1970), *Immunology*, 18, 645.
- FROMMEL, D., PEREY, D.Y. and GOOD, R.A., (1970), *J. Immun.*, 105, 1.
- FULLER, R. and JAYNE-WILLIAMS, D.J., (1970), *Res. vet. Sci.*, 11, 363.
- GALLAGHER, J.S. and VOSS, E.W.J., (1969), *Immunochemistry*, 6, 199.
- GENGOZIAN, N. and WOLFE, H.R., (1957), *J. Immun.*, 78, 401.
- GLEESON-WHITE, M.H., HEARD, D.H., MYNORS, L.S. and COOMBS, R.R.A., (1950), *Brit. J. exp. Path.*, 31, 321.
- GLICK, B., (1960), *Poult. Sci.*, 39, 1097.
- GLICK, B., CHANG, T.S. and JAAP, R.G., (1956), *Poult. Sci.*, 35, 224.
- GLICK, B. and WHATLEY, S., (1967), *Poult. Sci.*, 46, 1587.
- GOOD, R.A., DALMASSO, A.P., MARTINEZ, C., ARCHER, O.K., PIERCE, J.C. and PAPERMASTER, B.W., (1962a), *J. exp. Med.*, 116, 773.
- GOOD, R.A., GABRIELSEN, A.E., PETERSON, R.D.A., FINSTAD, J. and COOPER, M.D., (1966), in *Ciba Foundation Symposium*

- "The Thymus: experimental and clinical studies", ed. by Wolstenholme, G.E.W. and Porter, R., Churchill, London, p.188.
- GOOD, R.A., KELLY, W.D., RÖTSTEIN, J. and VARCO, R.L., (1962b), *Prog. Allergy*, 6, 187.
- GOODMAN, M. and RAMSEY, D.S., (1957), *Fedn Proc. Fedn Am. Socs exp. Biol.*, 16, 416.
- GOODMAN, M., WOLFE, H.R. and NORTON, S., (1951), *J. Immun.*, 66, 225.
- GOUDIE, R.B., HORNE, C.H.W. and WILKINSON, P.C., (1966), *Lancet* II, 1224.
- GOWMAN, J.L. and KNIGHT, E.J., (1964), *Proc. R. Soc.*, 159, 257.
- GRABAR, P. and WILLIAMS, C.A., (1953), *Biochim. Biophys. Acta*, 10, 193.
- GRAETZER, M.A., COTE, W.P. and WOLFE, H.R., (1963), *J. Immun.*, 91, 576.
- GRAETZER, M.A., WOLFE, H.R., ASPINALL, R.L. and MEYER, R.K., (1963), *J. Immun.*, 90, 878.
- GREAVES, M.F., (1970), *Transplant. Rev.*, 5, 45.
- GREAVES, M.F., MÖLLER, E. and MÖLLER, G., (1970), *Cell. Immunol.*, 1, 386.
- HEKTOEN, L., (1918), *J. infec. Dis.*, 22, 561.
- HERSH, R.T. and BENEDICT, A.A., (1966), *Biochim. Biophys. Acta (Aust.)*, 115, 242.
- HILL, W.C., (1971), *J. Immun.*, 106, 414.
- HILL, W.C. and NISSEN, B., (1971), *J. Immun.*, 106, 421.

HUMPHREY, J.H., PARROTT, D.M.V. and EAST, J., (1964),
Immunology, 7, 419.

ISAKOVIC, K. and JANKOVIC, B.D., (1964), Int. Archs
Allergy appl. Immun., 24, 296.

IVANYI, J., MARVANOVÁ, H. and SKAMENE, E., (1969),
Immunology, 17, 325.

IVANYI, J., SKAMENE, E. and KURISU, A., (1970), Folia
biol., Praha, 16, 34.

IVANYI, J., VALENTOVA, V. and CERNY, J., (1966), Folia
biol., Praha, 12, 157.

JANKOVIC, D. and ISAKOVIC, K., (1966), Nature, Lond.,
211, 202.

JANKOVIC, D. and ISAKOVIC, K., (1967), Folia biol., Praha,
13, 401.

JANKOVIC, B.D. and ISVANESKI, M., (1963), Int. Archs
Allergy appl. Immun., 23, 188.

JANKOVIC, B.D., ISVANESKI, M., MILOSEVIC, D. and POPESKOVIC,
L., (1963), Nature, Lond., 198, 298.

JANKOVIC, B.D. and LESCOWITZ, S., (1965), Proc. Soc. exp.
Biol. Med., 118, 1164.

JANKOVIC, B.D. and MITROVIK, K., (1967), Folia biol., Praha,
13, 406.

JANKOVIC, B.D., WAKSMAN, B.H. and ARNASON, B.G., (1962),
J. exp. Med., 116, 159.

JERNE, N.K. and NORDIN, A.A., (1963), Science, N.Y.,
140, 405.

- JOLLY, J., (1909), CR Soc. Biol. Paris, 66, 499.
- JONES, G., TORRIGIANI, G. and ROITT, I.M., (1971),
J. Immun., 106, 1425.
- KEILY, D. and ABRAMOFF, P., (1969), J. Immun., 102, 1058.
- KERSTETTER, T.H., BUSS, I.O. and WENT, H.A., (1962),
J. exp. Zool., 149, 233.
- KINCADE, P.W. and COOPER, M.D., (1971), J. Immun., 106,
371.
- KINCADE, P.W., LAWTON, A.R., BOCKMAN, D.E. and COOPER,
M.D., (1970), Proc. natn. Acad. Sci. U.S.A., 67, 1918.
- KINCADE, P.W., LAWTON, A.R. and COOPER, M.D., (1971),
J. Immun., 106, 1421.
- KLEMPERER, T., (1893), Arch. exp. Path. Pharmacol., 31, 356.
- KOHN, H.I., (1950), J. Immun., 66, 525.
- KONO, R., AKAO, Y., SASAGAWA, A. and NOMURA, Y., (1969),
Jap. J. med. Sci. Biol., 22, 235.
- KUBO, R.T. and BENEDICT, A.A., (1969), J. Immun., 102, 1523.
- LEBACQ-VERHEYDEN, A., VAERMAN, J.P. and HEREMANS, J.F.,
(1972), Immunology, 22, 165.
- LESLIE, G.A. and CLEM, L.W., (1969), J. exp. Med., 130,
1337.
- LINNA, T.J., FROMMEL, D. and GOOD, R.A., (1972), Int.
Archs Allergy appl. Immun., 42, 20.
- LIPTON, M.M. and STEIGMAN, A.J., (1961), J. Immun., 86,
445.

- MAKINODAN, T., GENGOZIAN, N. and CANNING, R.E., (1960),
J. Immun., 85, 439.
- MAKINODAN, T., RUTH, R.F. and WOLFE, H.R., (1954),
J. Immun., 72, 45.
- MALKINSON, M., (1965), Immunology, 9, 311.
- MANCINA, G., CARBONARA, A.D. and HEREMANS, J.F., (1965),
Immunochemistry, 2, 235.
- MARVANOVA, H. and HAJEK, P., (1969), Folia biol., Praha,
14, 171.
- MASON, S. and WARNER, N.L., (1970), J. Immun., 104, 762.
- METCALF, D., (1956), Br. J. Cancer, 10, 442.
- MEYER, R.K., RAO, M.A. and ASPINALL, R.L., (1959),
Endocrinology, 64, 890.
- MILLER, J.F.A.P., (1961), Lancet, II, 748.
- MILLER, J.F.A.P., (1962a), Proc. R. Soc., 156, 415.
- MILLER, J.F.A.P., (1962b), Adv. Immunol., 2.
- MILLER, J.F.A.P., (1963), Lancet, I, 43.
- MILLER, J.F.A.P., (1965), Br. med. Bull., 21, 111.
- MILLER, J.F.A.P., (1966), in Ciba Foundation Symposium
"The Thymus: experimental and clinical studies", ed. by
Wolstenholme, G.E.W. and Porter, R., Churchill, London,
p.153.
- MILLER, J.F.A.P., DOAK, S.M.A. and CROSS, A.M., (1963),
Proc. Soc. exp. Biol. Med., 112, 785.
- MILLER, J.F.A.P. and OSOBA, D., (1963), in "The
Immunologically Competent Cell", Ciba Study Group No. 16,
ed. by Wolstenholme, G.E.W. and Knight, J., Churchill,
London, p.62.

- MITCHELL, G.F. and MILLER, J.F.A.P., (1968), *Proc. natn. Acad. Sci., U.S.A.*, 59, 296.
- MITCHISON, N.A., (1967), *Cold Spring Harbor Symp. quant. Biol.*, 32, 431.
- MITCHISON, N.A., (1968), *Symp. Int. Soc. Cell. Biol.*, 7, 29.
- MOLLER, G., (1970), in *"Immune Surveillance"*, ed. by Smith, R.T. and Landy, M., Academic Press, N.Y., p.85.
- MOORE, M.A.S. and OWEN, J.J.T., (1966), *Devl Biol.*, 14, 40.
- MUELLER, A.P., SATO, K. and GLICK, B., (1971), *Cell. Immunol.*, 2, 140.
- MUELLER, A.P., WOLFE, H.R. and COTE, W.P., (1964), in *"The Thymus in Immunobiology"*, ed. by Good, R.A. and Gabrielsen, A.E., Hoeber, N.Y., p.359.
- MUELLER, A.P., WOLFE, H.R. and MEYER, R.K., (1960), *J. Immun.*, 85, 172.
- MUELLER, A.P., WOLFE, H.R., MEYER, R.K. and ASPINALL, R.L., (1962), *J. Immun.*, 88, 354.
- NETER, E., WESTPHAL, O., LÜDERITZ, E.A., GORZYNSKI, E.A. and EICHENBERGER, E., (1956), *J. Immun.*, 76, 377.
- NICHOL, J.C. and DEUTSCH, H.F., (1948), *J. Am. chem. Soc.*, 70, 80.
- ORLANS, E., (1967), *Immunology*, 12, 27.
- ORLANS, E., (1968), *Immunology*, 14, 61.
- ORLANS, E., ROSE, M.E. and MARRACK, J.R., (1961), *Immunology*, 4, 262.

OSOBA, D. and MILLER, J.F.A.P., (1963), *Nature, Lond.*, 199, 653.

OUCHTERLONY, O., (1953), *Acta path. microbiol. scand.*, 32, 231.

PAPERMASTER, B.W. and GOOD, R.A., (1962), *Nature, Lond.*, 196, 838.

PARASKEVAS, F., LEE, S.T. and ISRAELS, L.G., (1971), *J. Immun.*, 106, 160.

PATTERSON, R., SUSZKO, I.M. and PRUZANSKY, J.J., (1965), *Proc. Soc. exp. Biol. Med.*, 118, 307.

PATTERSON, R., YOUNGER, J.S., WEIGLE, W.O. and DIXON, F.J., (1962), *J. Immun.*, 89, 272.

PAYNE, L.N. and RENNIE, M., (1970), *J. Natn. Cancer Inst.*, 45, 387.

PENHALE, W.J., LOGAN, E.F. and STENHOUSE, A., (1971), *Vet. Rec.*, 89, 623.

PEREY, D.Y.E., DUPUY, J.M. and GOOD, R.A., (1970a), in "Developmental Aspects of Antibody Formation and Structure", ed. by Sterzl, J. and Riha, I., Academic Press, N.Y., p.171.

PEREY, D.Y.E., FROMMEL, D., HONG, R. and GOOD, R.A., (1970b), *Lab. Invest.*, 22, 212.

PETERSON, R.D.A., BURMEISTER, B.R., FREDRICKSON, T.N., PURCHASE, H.G. and GOOD, R.A., (1964), *J. Natn. Cancer Inst.*, 32, 1343.

PIERCE, H.E., CHUBB, R.C. and LONG, P.L., (1966), *Immunology*, 10, 321.

- STEDMAN, R.A., SINGLETON, L. and BOX, P.G., (1969),
J. Comp. Path. Ther., 79, 507.
- STEINBERG, S.U., MUNRO, J.A., FLEMING, W.A., FRENCH, V.I.,
 STARK, J.M. and WHITE, R.G., (1970), *Immunology*, 18, 635.
- STERZL, J., (1966), *Nature, Lond.*, 209, 416.
- STERZL, J. and SILVERSTEIN, A.M., (1967), *Adv. Immunol.*,
 6, 337.
- SUTHERLAND, W.D. and MITRA, G.C., (1914), *Indian J. med.
 Res.*, 1, 707.
- SZENBERG, A., LIND, P. and CLARKE, K., (1965), *Aust. J.
 exp. Biol. med. Sci.*, 43, 451.
- TALIAFERRO, W.H., TALIAFERRO, L.G. and JANSSEN, E.F.,
 (1952), *J. infec. Dis.*, 91, 105.
- TENENHOUSE, H.S. and DEUTSCH, H.F., (1966), *Immunochemistry*,
 3, 11.
- THORBECKE, G.J., GORDON, H.A., WOSTMAN, B., WAGNER, M.
 and REYNIERS, J.A., (1957), *J. infec. Dis.*, 101, 237.
- UHR, J.W., FINKELSTEIN, M.S. and FRANKLIN, E.C., (1962),
Proc. Soc. exp. Biol. Med., 111, 13.
- VAN ALTEN, P.J., CAIN, W.A., GOOD, R.A. and COOPER, M.D.,
 (1968), *Nature, Lond.*, 217, 358.
- VAN METER, R., GOOD, R.A. and COOPER, M.D., (1969),
J. Immun., 102, 370.
- VAN ORDEN, D. and TREFFERS, H., (1968), *J. Immun.*, 100, 664.

- WAKSMAN, B., (1959), *Int. Archs Allergy appl. Immun.*, 14, Suppl.
- WAKSMAN, B.H., ARNASON, B.G. and JANKOVIC, B.D., (1962), *J. exp. Med.*, 116, 187.
- WARNER, N.L., (1965), *Aust. J. exp. Biol. med. Sci.*, 43, 439.
- WARNER, N.L., OVARY, Z. and KANTOR, F.S., (1971), *Int. Archs Allergy appl. Immun.*, 40, 719.
- WARNER, N.L. and SZENBERG, A., (1964), in "The Thymus in Immunobiology", ed. by Good, R.A. and Gabrielsen, A.E., Hoeber, N.Y., p.395.
- WARNER, N.L. and SZENBERG, A., (1962), *Nature, Lond.*, 196, 784.
- WARNER, N.L., SZENBERG, A. and BURNET, F.M., (1962), *Aust. J. exp. Biol. med. Sci.*, 40, 373.
- WARNER, N.L., UHR, J.W., THORBECKE, G.J. and OVARY, Z., (1969), *J. Immun.*, 103, 1317.
- WEBER, W.T. and WEIDANZ, W.P., (1969), *J. Immun.*, 103, 537.
- WILKINSON, P.C. and FRENCH, V.I., (1969), *Immunochemistry*, 6, 498.
- WOLFE, H.R., (1942), *J. Immun.*, 44, 135.
- WOODS, R. and LINNA, J., (1965), *Acta path. microbiol. scand.*, 64, 470.